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SYNTHESIS OF TRIAZENES AND THE STUDY OF THEIR CHEMICAL REACTIONS WITH NUCLEIC ACIDS

by

© RANJIT SINGH

A THESIS

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Date June 29 1981

ABSTRACT

A series of novel 3-(2-haloethyl)aryltriazenes, many of which exhibit marked antileukemic activity in animal test neoplasms, has been synthesized and characterized both spectroscopically and by their esterification of 3,5-dinitrobenzoic acid. These compounds can be prepared by aqueous condensation of an aryl diazonium cation with the appropriate 2-haloethylamine.

Their rates of decomposition in aqueous media were determined polarographically by monitoring the electrochemically active triazene moiety. The rates of decomposition were found to increase markedly with lowered pH in the range 9.3 to 4.65. The products of aqueous decomposition, both volatile and involatile, were identified and quantified by GC and GC/MS. These included 1-(p-cyanophenyl)-3-(2-chloroethyl)triazene, 1-(p-cyanophenyl)-3-(2-chloropropyl)triazene and 5-[3-(2-fluoroethyl)-1-triazenyl]imidazole-4-carboxamide. Discrimination between alternative pathways of decomposition was possible by the preparation and use of selectively deuterated 1-(p-cyanophenyl)-3-(2-chloro-1,1-dideuterioethyl)triazene and 1-(p-cyanophenyl)-3-(2-chloro-2,2-dideuterioethyl)triazene. The observations are consistent with decomposition of 2-haloethyltriazene to generate the 2-haloethyl cation (or its kinetic equivalent). This cation is subject to rearrangements which are detected by deuterium scrambling. A second competing pathway which

was revealed by a specific deuterium labelling study may involve cyclization of the triazene to a 1-aryl-1,2,3-triazoline intermediate which then undergoes nucleophilic cleavage with attendant loss of nitrogen.

2-Haloethyltriazenes in general react readily with DNA under physiological conditions. In contrast to the base promoted decompositions of the related 2-haloethylnitrosoureas (which react largely by base alkylation of DNA) the triazenes, owing to their unique acid promoted decomposition, showed a preference for reaction at the more acidic phosphate sites. 1-(p-Cyanophenyl)-3-(2-chloroethyl)triazene and 5-[3-(2-fluoroethyl)-1-triazenyl]imidazole-4-carboxamide readily esterify diethyl phosphate. 1-(p-Cyanophenyl)-3-(2-chloroethyl)triazene degraded poly A by phosphate alkylation at a rate much faster than for the corresponding bis-(2-chloroethyl)nitrosourea. 2-Hydroxyethyltriazenes in general readily degraded DNA presumably by phosphate alkylation.

A series of antitumor 1-aryl-3-{S-(2-chloroethyl)thioethyl}triazenes has been synthesized as transport forms of sulfur mustard. For 1-(p-cyanophenyl)-3-{S-(2-chloroethyl)-thioethyl}triazene the rate of decomposition in aqueous medium, determined polarographically, increased markedly with decreasing pH in the range 7.1 to 5.1. The same triazene decomposed in aqueous solution to give bis-(2-hydroxyethyl)sulfide as the major volatile product. Synthesis and controlled decomposition of the selectively deuterated

1-(p-cyanophenyl)-3-{S-(2-chloroethyl)-1,1-dideuteriothioethyl}triazene permitted discrimination between alternative decomposition pathways. The observed isotope label scrambling indicated the intermediacy of an episulfonium species in this decomposition. Indication of a second competing pathway implicating a triazoline type intermediate was also obtained. Alkylation of uridine, chosen as a model for DNA base centres, by 1-(p-cyanophenyl)-3-{S-(2-chloroethyl)-thioethyl}triazene was observed to occur at the N^3-position of the uridine. 1-Aryl-3-{S-((2-chloroethyl)thioethyltriazenes also readily esterify diethyl phosphate. Since such a reaction may have a bearing on the cytotoxicity of triazenes, the mechanism of the esterification of 3,5-dinitrobenzoic acid has been studied using specifically deuterated triazenes. It may be concluded that the triazenes that generate unstable carbonium ions esterify largely by an S_N2 displacement mechanism whereas the generation of stable carbonium ions contributes increasingly to an ion-pair mechanism.

Sulfur mustard triazenes, e.g. 1-(p-cyanophenyl)-3-{S-(2-chloroethyl)thioethyltriazene, produce interstrand cross-links in DNA. The cross-linking was observed to increase with decreasing pH in the range 10-5. Different chemical reactivity during the alkylation of DNA by 1-(p-cyanophenyl)-3-{S-(2-chloroethyl)thioethyl}triazene and the structurally related 1-{2-[(2-chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea was observed. No depurination was

observed with the former whereas the latter produced extensive depurination as detected by the application of apurinic site specific enzyme, endonuclease VI.

A series of vinyltriazenes without substituents on the vinyl group has been synthesized and studied spectroscopically. In the case of 1-phenyl-3-vinyltriazene and 1-(2,5-dimethoxyphenyl)-3-vinyltriazene thermally and chemically induced isomerizations to the corresponding 1-aryl-3-ethylidinetriazene were observed. An examination of the products of aqueous decomposition of vinyltriazenes indicated the intermediacy of vinyl cations. The rates of the aqueous decompositions were determined polarographically. They indicated that the electron-withdrawing groups on the phenyl ring stabilize the triazeno-function relative to the electron-releasing groups.

Arylvinyltriazenes were observed to alkylate DNA under physiological conditions. The base alkylation of DNA by these types of vinyltriazenes, and the concomitant degradation of the DNA by the aromatic amines which are among the products of aqueous decomposition, have been observed largely by employing certain ethidium fluorescence assays.

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GLOSSARY

1 AIC 1-Aminoimidazole-4-carboxamide

2 BCNU Bis-(2-chloroethyl)-1-nitrosourea

3 BIC 5-[3,3-Bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide

4 DIC 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide

5 MIC 5-(3-Monomethyl-1-triazeno)imidazole-4-carboxamide

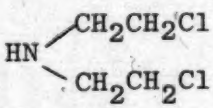
6 NBP γ-(p-Nitrobenzyl)pyridine

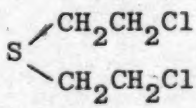
7 PDT 3,3-Dimethyl-1-phenyltriazene

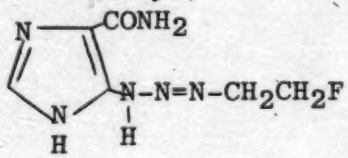
8 PMT 3-Methyl-1-phenyltriazene

9 Poly(A) Polyadenylic Acid

10 Poly(G) Polyguanylic Acid

11 Nitrogen mustard 

12 Sulfur mustard 

13 5-{3-(2-Fluoroethyl)-1-triazenyl}imidazole-4-carboxamide 

14 The terms "2-haloalkyltriazene" and "2-haloalkylaryltriazene" have been used in this thesis to denote 2-halogenoalkylaryltriazene.

15	CIMS	Chemical Ionization/Mass Spectrometry
16	GCMS	Gas Chromatography/Mass Spectrometry
17	Ir	Infrared spectrum
18	Pmr	Proton magnetic spectrum

19	AP	Apurinic and apyrimidinic
20	CCC	Covalently closed circular
21	CLC	Covalently closed complementary
22	SSS	Single strand scission

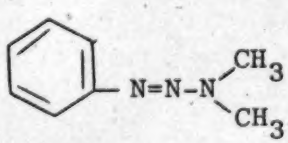
23 It is realized that the widely accepted unit to express the concentration of polynucleic acids is OD but in this thesis A_{260} has been used for practical purposes.

INTRODUCTION

Triazenes have experienced long-standing use in the synthesis of azo dyes, in the rubber industry, and in the production of high-octane gasoline. Tumor-inhibitory activity of a triazene, a phenyl derivative, was first reported by Clarke *et al.* in 1955.[1] Subsequently, imidazole triazenes with potential anti cancer activity [5-substituted triazeno imidazole-4-carboxamides] were synthesized by Shealy *et al.*[2,3] Thus two main lines of triazenes have been pursued for antitumor therapy: (1) phenyltriazenes, and (2) imidazole triazenes.

Phenyltriazenes

The growth inhibitory activity of 3,3-dimethyl-1-phenyltriazene (PDT) 1 in subcutaneous mouse sarcoma 180, as reported by Clarke *et al.*,[1] was initially attributed to the formation of a diazonium ion.

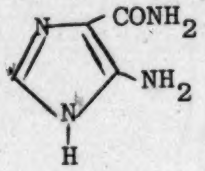


1

This compound proved to be the most promising of a large series of compounds tested, mostly triazene derivatives.[4]

Imidazole Triazenes

The ribotide of aminoimidazolecarboxamide (AIC) 2 is a precursor in purine biosynthesis. In an attempt to



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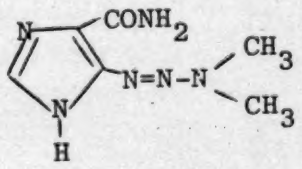
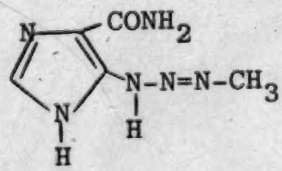
design antagonists of AIC that could be used in cancer chemotherapy, Shealy *et al.* derivatized AIC as 1-triazeno imidazole-4-carboxamides.[3,5,6] The prototype of triazene compounds 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DIC) 3 revealed tumor inhibition in mouse leukemia L1210, Sarcoma 180, and adenocarcinoma 755[3] and was selected for



3

clinical evaluation. DIC and its derivatives also inhibited Walker carcinosarcoma of rats[7] and a human malignant melanoma. DIC was found to be more stable in aqueous and alcoholic solutions than its demethylation product, 5-(3-monomethyl-1-triazeno)imidazole-4-carboxamide (MIC) 4 which releases AIC during decomposition.[8] Screening of



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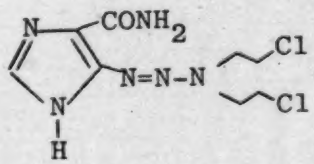
large numbers of triazene derivatives in L1210 murine leukemia revealed that these compounds, with at least one methyl group (of the two alkyl groups), are the most effective ones.[8]

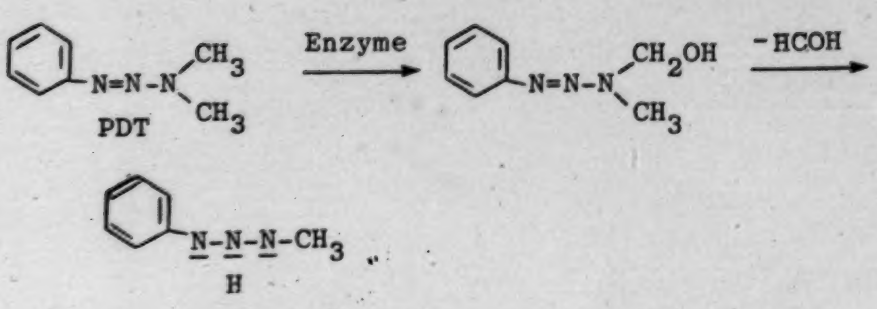
The hazards of structure-activity evaluations and speculations are evidenced by the fact that 5-[3,3-bis (2-chloroethyl)-1-triazeno]-imidazole-4-carboxamide (BIC) 5 was the most potent derivative in L-1210 mouse leukemia,



5

both in the initial form[9] and in the advanced form.[10] Also, it is now generally accepted that DIC does not act as an antagonist of AIC and that its anti-tumor action is a property only of its triazeno function. It has been shown that triazenes not containing the imidazole ring are just as effective antitumor agents.[11,12]

The first insight into the mechanism of biological activity of dimethyltriazene PTD was obtained through the studies of Preussman *et al.*[13] which revealed that PDT is enzymatically demethylated to 3-methyl-1-phenyltriazene (PMT) with formation of formaldehyde (Scheme 1).



Scheme 1

PMT then acts like an alkylating agent, e.g. transferring a methyl group to guanine residues to form 7-methylguanine in the RNA and DNA[14] of liver in rats treated *in vivo* with PTD. Skibba *et al.*,[15] studying the metabolism of DIC in rats, found oxidative N-demethylation of DIC *in vitro* by rat liver microsomes, as well as *in vivo*, after intraperitoneal injection of ^{14}C-methyl-labelled DIC. The resulting MIC spontaneously decomposes to AIC and a methylating agent. Subsequently, transmethylation of the methyl group of MIC on to the 7 position of guanine of rat liver DNA and RNA takes place.

These observations bring out a very important aspect of the mechanism of biological activity of triazenes in general, namely the existence of a correlation between their cytotoxicity and their ability to alkylate macromolecular cell components especially polynucleic acids. It is also evident that monoalkyl triazenes are the precursors of alkylating species. Monoalkyl triazenes exhibit carcinogenic,[13] mutagenic,[16] antifungal[17] and antitumor activity.[18]

Previous work involving the relationships of the chemical properties to biological properties resulted in the suggestions that the alkylating portion of the triazene is responsible for therapeutic effects[19,20] and the released aromatic amine involved in methemoglobinemia is related to toxicity effects.[13] Another important aspect of triazenes as prospective therapeutic drugs, namely lipophilicity, has not been investigated. While all three aspects appear important for physiological activity, it was the correlation between therapeutic activity and alkylating ability of the triazenes that was instrumental in initiating the work presented in this dissertation.

Many of the effects of alkylating agents in biological systems at the molecular level are not well understood. They react with several macromolecular cell components and produce a number of biochemical effects. It is being recognized that many clinically useful agents have nucleic acids as their principal cell target sites and consequently degrade DNA as one aspect of their mechanism of action. These include mitomycins B and C,[21] nitrosoureas[22,23] and triazenes.[24]

A general equation for alkylation can be written as follows:

$$\text{Nuc.} + \text{R-X} \longrightarrow \text{R-Nuc.} + \text{X}^-$$

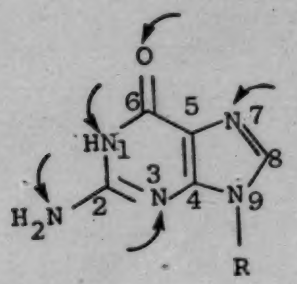
Nuc. is the nucleophile which is alkylated and R is an alkyl group attached to a leaving group X. The mechanism of an

alkylation reaction can be either S_N2 process or an S_N1 process. Simultaneous occurrence of both mechanisms is possible.

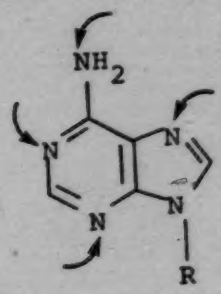
A number of factors may influence the course of a particular reaction. If the process of alkylation involves charged transition states or intermediates, polar solvents such as water will tend to lower activation energies and stabilize intermediates. Similar reactions occurring in nonpolar solvents will be considerably slower. Neighboring groups can play an important role in assisting the displacement of X from R and producing stabilized intermediates which react as alkylating agents. Typical examples involving neighboring group participation include the sulfur and nitrogen mustards where chemically reactive three membered aziridinium and episulfonium ions, respectively, are produced.

The products resulting from DNA alkylation depend upon the nature as well as the source of the alkylating species in conjunction with the relative reactivities of various sites on the DNA macromolecule.[25] The nucleophilic sites in DNA potentially resulting in base alkylation are shown on the following page. The 7-position of guanosine is one of the most readily alkylated sites, especially by S_N2 alkylating agents. 7-Alkylguanosine may account for 90% of the total base substitution.[26] A number of other sites, including the 1, 3 and 7 positions of adenosine and the 3 position of cytidine, have also been shown to react with

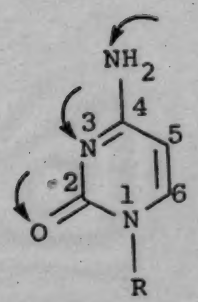
alkylating agents.[27]



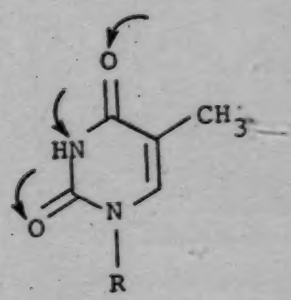
Guanosine



Adenosine



Cytidine



Thymidine

The structures of the nucleosides shown are the accepted major tautomeric forms observed in aqueous solution. The energies and alkylations of tautomeric heterocyclic compounds have been reviewed.[28] This led to the suggestion that the extrapolation of tautomeric equilibrium constants from one molecular environment to another is inapplicable.[28]

The energies of tautomerization can be greatly influenced by local molecular environment. This may lead to "rare" tautomeric forms of purines and pyrimidines in base paired hydrophobic areas of the DNA duplex and the extent of "rare" tautomeric forms can not be assessed on the basis of aqueous studies.

It has also been suggested that if the factors which determine the ground state energy difference between tautomers, also control the relative transition-state energies for the first step of an alkylation, then the product formed will have the alkyl group attached to the heteroatom which does not bear the proton in the major tautomer.[28]

The biological implications of alkylation of DNA at various sites have been investigated. A recent study shows that a number of minor DNA alkylation products may be biologically more significant than alkylation at N-7 of guanosine.[29] It has been reported that N-7 methylated poly G permits incorporation of cytidine residues in the same manner as does poly G.[29] Alkylation of the O-6 position of guanosine has been reported.[30] This reaction, in addition to cytidine N-3 alkylation, might result in significant mispairing and miscoding of bases. Alkylation of other sites such as N-3 position of guanosine and O-4 position of thymidine,[31,32] O-2 position of cytidine and nearly every potentially nucleophilic site of polyuridylic acid including the 2'-O position of the ribose has been documented.[33-35]

In addition to base alkylation, there is good evidence that DNA is alkylated at the phosphodiester group to varying extents. Phosphate alkylation represents 15% of the total alkylation when DNA is treated with ethyl methanesulfonate and only 1% when treated with methyl methanesulfonate. Work with poly A[36] and dideoxynucleotides[37] has shown indirectly that esterification of phosphates does occur significantly with ethylating agents. Depending upon the nature of the alkylating agent, phosphate alkylation may become a major event and may play a significant role in therapeutic activity since the phosphotriesters formed can bring about degradation of DNA in the form of single strand breaks.[27]

Bifunctional alkylating agents can undergo a second alkylation after initial attachment to the DNA. Bifunctional alkylating agents have generally been observed[26] to be more cytotoxic than their monofunctional counterparts. The formation of interstrand and/or intrastrand DNA cross-links between two guanine residues for example in the case of sulfur mustard has been observed by Brookes and Lawley.[38] Interstrand DNA cross-links induced by other bifunctional alkylating agents have been detected using various techniques such as reversible denaturation experiments,[39] spectrofluorometric assays[40] and inhibition of alkali-induced strand separation.[41]

Many of the alkylating agents are related to mustard gas, first used for chemical warfare in World War I.

At that time it was noted[27] that limited exposure to this agent caused bone marrow suppression somewhat similar to that produced by radiation. Following this observation, a group at Yale University tried a nitrogen mustard, tris chloroethylamine, for the treatment of certain bone marrow-related malignancies. These studies, which were undertaken during World War II and described afterward by Gilman,[42] not only established the clinical potential of the mustards, but also demonstrated their disadvantages i.e. toxicity to the host and development of resistance by the tumor. Consequently, a wide search was initiated for new and more selective alkylating agents.

In the last decade or so, there has been somewhat less emphasis on alkylating agents than on other classes of compounds, e.g. DNA intercalators, or site-specific radical generators, possibly because of an impression that all the alkylating agents may be similar in their mechanism of action. Although in this point of view this is understandable, some important differences between alkylating agents and in the characteristics of normal and neoplastic tissue have been recognized. It seems entirely possible that these differences can be exploited further. For example the selective acid promoted decomposition of monoalkyltriazenes combined with the characteristic of neoplastic tissue to exhibit somewhat lower pH than normal tissue[43,44] may be sited as an example of selectivity. Several mono-

alkylaryltriazenes with structural variations both in the alkylating moiety as well as in the aromatic ring were synthesized during this study and submitted for *in vivo* studies. The *in vivo* data indicated that many of these novel triazenes are potent antitumor agents. The subsequent objectives decided upon in an attempt to understand the chemical mechanisms by which these triazenes exerted their antitumor effects were threefold. An investigation of the products of aqueous decomposition was undertaken to assist in determining the reactive intermediates involved. The *in vitro* alkylation of DNA by antitumor nitrosoureas has been observed to correlate with their therapeutic effects.[45] A similar study of DNA alkylation with triazenes was carried out using techniques including a sensitive ethidium bromide fluorescence assay. Finally, depending upon the nature of alkylating species released from a triazene, the fate of alkylated DNA such as its degradation or interstrand cross-linking has been investigated.

Since reactions of aryltriazenes with DNA are discussed extensively in this thesis it is appropriate to append a summary of the basic chemistry, stereochemistry and topology of the different DNAs employed in this research (see Appendix).

A brief discussion of the relevant work as it applies to successive aspects of this study will introduce each of the subsequent chapters.

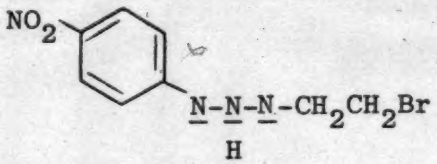
CHAPTER II.

STUDIES RELATED TO THE SYNTHESIS AND AQUEOUS DECOMPOSITION OF 2-HALOALKYLARYLTRIAZENES

Only one example of a mono(2-haloethyl)triazene, namely 5-{3-(2-chloroethyl)-1-triazenyl}imidazole-4-carboxamide 6, has been reported [46] together with a brief mention of its aqueous decomposition. Since this did not provide adequate information upon which to base our projected chemical and biochemical studies related to this class of compounds, it became necessary to conduct a systematic investigation into the basic chemistry of 2-haloalkyltriazenes. The information thus obtained was used to interpret the reactions of these compounds with DNA. The objective was to delineate those structural modifications to optimize their antitumor properties.

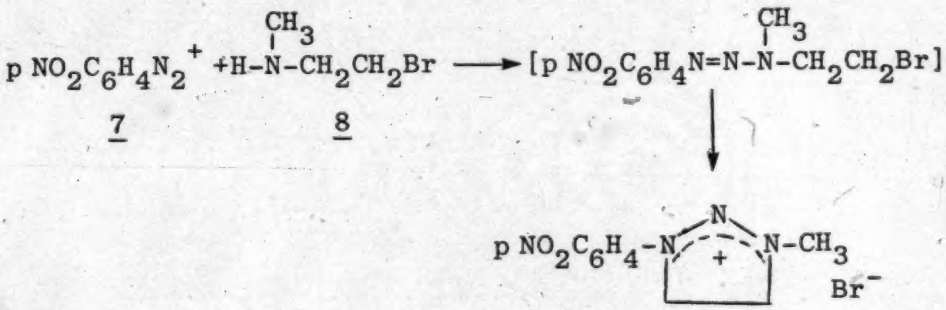
The aryltriazenes required in this study were prepared by coupling of the aryl diazonium cation with the 2-haloethylamine.[47] The observation that the presence of an electron-withdrawing group in the phenyl ring, particularly at the p-position, increases the stability of the triazene and prevents formation of the pentaazadiene during the reaction of the aryldiazonium ion with the primary amine[48] was exploited in these preparations. In general it was found that a *p*-cyano substituent in the phenyl ring conferred the desired stability characteristics. A special comment may be made in the case of the 2-bromoethyltriazene

14. It was recently reported that when the *p*-nitrophenyl-

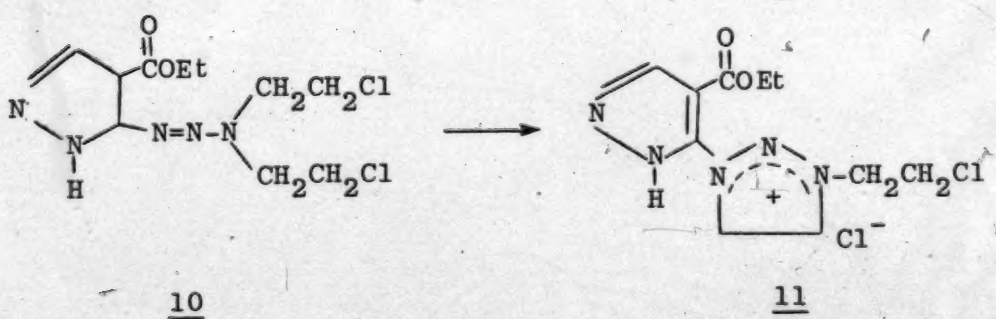


14

diazonium salt 7 was condensed with 2-bromoethylmethylamine 8 that the product was that of intramolecular cyclization to the triazolinium salt[49] 9. Similar triazolinium salts have been observed as a primary metabolite of BIC[50] and as a product of spontaneous cyclization of 10 to 11.[51]



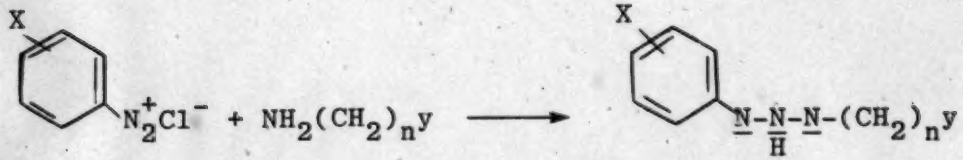
7 8 9



10 11

No such cyclization was observed in the preparation of the 2-bromoethyltriazene 14 that was obtained as a mixture of tautomers similar to the other triazenes. However cyclization to a 1-aryl-Δ^2-1,2,3-triazoline may be significant in the aqueous decomposition of triazenes as discussed later. The purification of 2-haloethyltriazenes required special care and was carried out by recrystallization at low temperatures. Purification by standard chromatographic methods was not possible owing to the rapid degradation on solid adsorbents as observed previously.[52] The composition, structures and purity of the triazenes were established by exact mass spectral measurements and by pmr and infrared spectroscopy. In several cases individual triazenes were characterized by their formation of ester derivatives from 3,5-dinitrobenzoic acid. The 2-haloethyltriazenes are thermally as well as chemically more labile than monoalkyltriazenes. Their instability increases in the sequence F<Cl<Br. It was also observed that the stability and reactivity is influenced by the number of methylene units intervening between the azide moiety and the halogen atom. The general synthetic procedure afforded the triazenes listed in Table 1. The stability of the triazeno function in 2-haloalkyltriazenes is mainly governed by the nature of the substituent and its position on the aromatic ring, and is also sensitive to the nature of the halogen in the alkyl group at the N-3 position. In common with other aryltriazenes, the 2-haloethyltriazenes were observed to

TABLE 1



x	y	n	Compound	
p-NO_2	F	2	1-(p-nitrophenyl)-3-(2-fluoroethyl)triazene	12
p-NO_2	Cl	2	1-(p-nitrophenyl)-3-(2-chloroethyl)triazene	13
p-NO_2	Br	2	1-(p-nitrophenyl)-3-(2-bromoethyl)triazene	14
p-CN	F	2	1-(p-cyanophenyl)-3-(2-fluoroethyl)triazene	15
p-CN	Cl	2	1-(p-cyanophenyl)-3-(2-chloroethyl)triazene	16
p-CN	Br	2	1-(p-cyanophenyl)-3-(2-bromoethyl)triazene	17
p-$COCH_3$	F	2	1-(p-acetylphenyl)-3-(2-fluoroethyl)triazene	18
p-$COCH_3$	Cl	2	1-(p-acetylphenyl)-3-(2-chloroethyl)triazene	19
p-$COOC_2H_5$	F	2	1-(p-ethoxycarbonylphenyl)-3-(2-fluoroethyl)triazene	20
p-$COOC_2H_5$	Cl	2	1-(p-ethoxycarbonylphenyl)-3-(2-chloroethyl)triazene	21

Table 1, continued

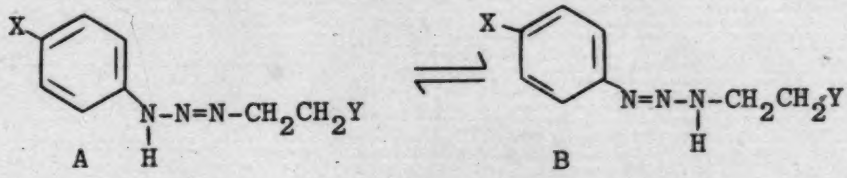
x	y	n	Compound	
o-NO_2	F	2	1-(o-nitrophenyl)-3-(2-fluoroethyl)triazene	22
o-NO_2	Cl	2	1-(o-nitrophenyl)-3-(2-chloroethyl)triazene	23
p-CN	Cl	3	1-(p-cyanophenyl)-3-(3-chloropropyl)triazene	24
p-CN	Cl	4	1-(p-cyanophenyl)-3-(4-chlorobutyl)triazene	25
p-CN	Cl	5	1-(p-cyanophenyl)-3-(5-chloropentyl)triazene	26
p-$COCH_3$	Cl	3	1-(p-acetylphenyl)-3-(3-chloropropyl)triazene	27

1-(p-cyanophenyl)-3-(2-chloro-1,1-dideuterioethyl)triazene 32

1-(p-cyanophenyl)-3-(2-chloro-2,2-dideuterioethyl)triazene 33

1-(p-cyanophenyl)-3-(2-chloropropyl)triazene 34

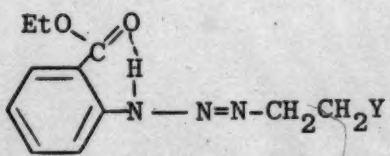
exist in solution as mixtures of tautomers. Since the position of equilibrium has a direct bearing on the mechanism of decomposition it was studied for each compound by pmr.



A B

$X = NO_2, CN, COCH_3, CO_2C_2H_5$ $Y = F, Cl, Br$

An electron-withdrawing group X in the *para* position strongly favours tautomeric form A, whereas such a substituent in the *ortho* position gives tautomer A exclusively; results which are similar to those for other types of triazenes.[53] 2-Haloethytriazenes with an electron-withdrawing group in the *ortho* position (and therefore in tautomeric form A) are much more labile than their *para* substituted counterparts. For example triazene 20 was isolable as a stable solid whereas its *ortho* isomer decomposed as soon as it was formed. A possible explanation in accord with the proposed mechanism of decomposition (discussed subsequently) is that the *ortho* triazene is fixed in tautomeric form A by hydrogen bonding:

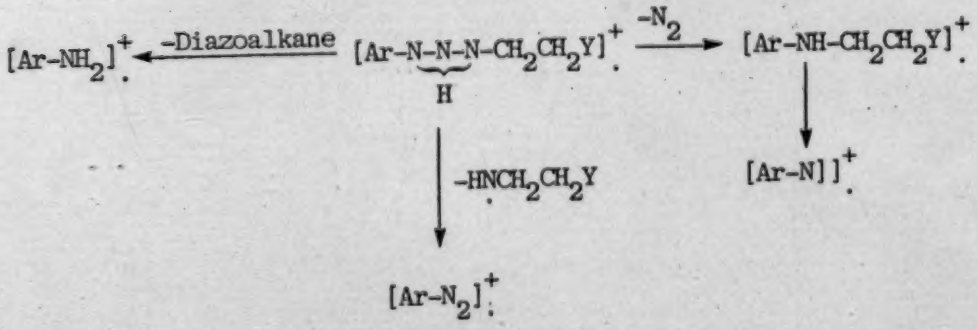


The resulting partial displacement of charge on to the aniline nitrogen may facilitate its protonation leading to decomposition.

The presence of either tautomer in the equilibrium mixtures was detected by its characteristic bands in the infrared and pmr spectra. For example the NH proton nmr signals corresponding to both tautomers are observable at -15°. The NH proton absorbing at higher field could be assigned to tautomer B on the basis of its coupling with the protons on the adjacent carbon at low temperature and the assigned couplings were confirmed by double resonance experiments.

The mass spectra of the 2-haloethyltriazenes showed several fragmentation patterns[47] which were useful in characterizing the new compounds as shown in Scheme 2.

Scheme 2



One new representative of the family of imidazole triazenes, namely 5-[3-(2-fluoroethyl)-1-triazenyl]imidazole-4-carboxamide 35 was synthesized for a comparison of its chemical properties with those of the 2-fluoroethylaryltri-

azenes. Triazene 35 was light sensitive and chemically labile. It was characterized by its spectral properties and by its reaction with 3,5-dinitrobenzoic acid to form 2-fluoroethyl-3,5-dinitrobenzoate.

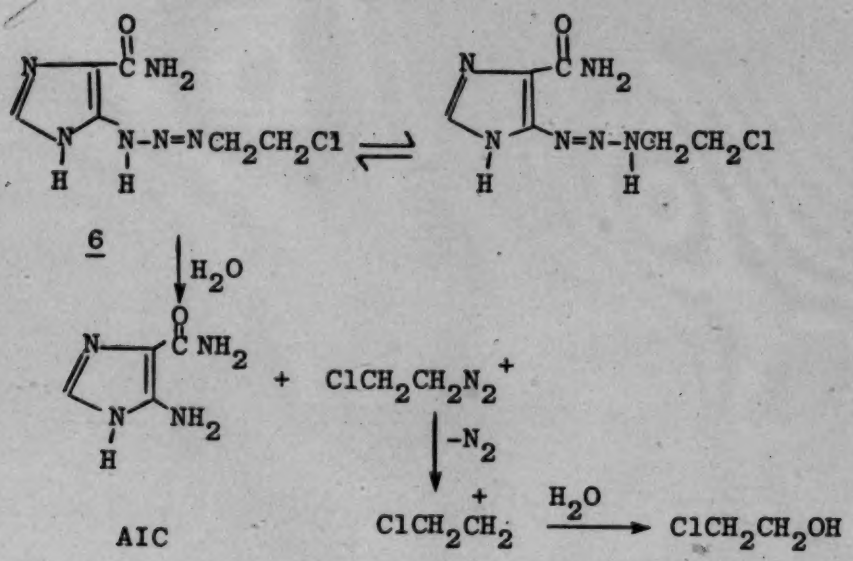
Certain specifically deuterated 2-haloethyltriazenes were required in order to establish the mechanistic pathways of formation of the products of decomposition. Accordingly triazenes 32 and 33 specifically deuterated on carbons C-1 and C-2, respectively were synthesized from the corresponding deuterated amines which were in turn prepared following the procedures of Brundrett *et al.*[54]

In 1975 Shealy *et al.* reported[46] 2-chloroethanol as the only identified volatile product of aqueous decomposition of mono(chloroethyl)triazene 6. AIC was reported as the major involatile product. The formation of two alkylated derivatives of AIC was also suggested on the basis of certain peaks in the mass spectra of the mixture of involatile products. However, no structures could be assigned.

As is evident from the scheme,[46] it was presumed that the decomposition of 6 proceeded exclusively via an S_N1 process. Moreover, 2-chloroethanol accounted for only 70% of the chloroethyltriazene 6, leaving 30% of the potential alkylating species unaccounted.

A group of six selected triazenes 15, 16, 32, 33 and 35 was separately allowed to decompose at 37° in phosphate buffered (0.1 M, pH 7.2) aqueous solution in gas-tight vials. In order to have an insight into the mechanism of

Scheme 3



decomposition of 2-haloalkyltriazenes, the volatile products were analyzed by gas chromatography (GC) and identified by gas chromatography/mass spectrometry (GC/MS) (Table 2). Each compound was identified by its retention time compared with an authentic sample and by its characteristic mass spectrum (see Table 2). The involatile products were separated by preparative thin layer chromatography (TLC) and identified spectrophotometrically. 1-(p-Cyanophenyl)-3-(2-chloroethyl)triazene 16 afforded acetaldehyde (11.5% of volatiles), 1,2-dichloroethane (11.5% of volatiles) and 2-chloroethanol (77% of volatiles). In addition, the following involatile products were identified: p-cyanoaniline (52% of involatiles); N(2-chloroethyl)-p-cyanoaniline (30%); 1,2-(p-cyanoanilino)ethane (10%), and N(2-hydroxyethyl)-p-cyanoaniline (8%). When this triazene 16 was allowed to

TABLE 2

MASS SPECTRAL IDENTIFICATION OF PRODUCTS OF AQUEOUS DECOMPOSITION OF PROTIUM AND SPECIFICALLY DEUTERIUM LABELLED 2-HALOETHYLARYLTRIAZENES

Triazene	Reaction Conditions	Decomposition Products	m/e (Relative Intensity, fragments)
21	phosphate buffer	CH_3CHO	44 (42.5, M^+); 43 (21.4, CH_3CO); 29 (100, CHO).
		$ClCH_2CD_2Cl$	100 (14.2, M^+ ^{35}Cl); 102 (9.0, M^+ ^{37}Cl); 64, 66 (100, 32.3, M^+ -HCl); 63, 65 (31.2, 28.6, M^+-DCl); 29 (79.3, C_2HD_2).
		$ClCH_2CD_2OH$	82 (3.6 M^+ ^{35}Cl), 84 (0.4 M^+ ^{37}), 33
		$ClCD_2CH_2OH$	100, CD_2OH), 31 (23.7, CH_2OH).
	buffer + KBr	$BrCH_2CD_2Cl$	144 (5.6, M^+ ^{35}Cl), 146 (7.0 M^+ ^{37}Cl),
		$BrCD_2CH_2Cl$	65 (100 M^+ -Br, ^{35}Cl), 67 (31.1 M^+ -Br ^{37}Cl),
22	phosphate buffer	CH_3CDO	45 (46.2, M^+), 43 (17.6, CH_3CO), 30 (100, CDO)
		$ClCH_2CD_2Cl$	100 (7.7, M^+ ^{35}Cl), 102 (4.1, M^+ ^{37}Cl), 64,66 (99.8, 29.6 M^+ -HCl); 63,65 (25.4, 25.4 M^+ -DCl), 29 (100, C_2HD_2).

Table 2., continued

Triazene	Reaction Conditions	Decomposition Products	m/e (Relative Intensity, fragments
		$ClCH_2CD_2OH$	82 (1.4, $M^{+35}Cl$), 84 (non visible, M^{+} ^{37}Cl),
		$ClCD_2CH_2OH$	31 (100, CH_2OH), 33 (3.4, CD_2OH)
	Buffer + KBr	$BrCH_2CD_2Cl$	144 (4.4, M^{+} ^{35}Cl), 146 (5.5, M^{+} ^{37}Cl)
		$BrCD_2CH_2Cl$	65 (100, M^{+} -Br ^{35}Cl) 67 (31.4, M^{+} -Br ^{37}Cl)
23	phosphate buffer	CH_3COCH_3	58 (24.9, M^{+}), 43 (100, M^{+}, -15)
		CH_3-CCl=CH_2	76 (43.7, M^{+} ^{35}Cl), 78 (14.8, M^{+} ^{37}Cl)
			39 (63.8, M^{+} ^{37}Cl), 41 (100, M^{+} ^{35}Cl)
		CH_3-CHCl-CH_2Cl	112 (4.9, M^{+} ^{35}Cl), 114 (3.0, M^{+} ^{37}Cl), 76, 78 (37.4, 12.7, M^{+} -HCl), 63, 65 (100, 29.8 M^{+} -CH_2Cl).
		CH_3-CHCl-CH_2OH	65 (3.7, M^{+} ^{35}Cl-CH_2OH), 67 (0.4, M^{+} ^{37}Cl -CH_2OH), 58 (11.7, M^{+} -HCl).
		CH_3-COH-CH_2Cl	31 (100, CH_2OH)

Table 2, continued

Triazene	Reaction Conditions	Decomposition Products	m/e (Relative Intensity, fragments
9 or 28	phosphate buffer	FCH_2CH_2F	66 (8.6, M^+), 46 (22.7, M^+ -HF), 33 (100, CH_2F).
		FCH_2CH_2OH	64 (12.4, M^+), 45 (15.7, M^+ -F), 31 (100, CH_2OH).
		$CH_3CHOH-CH_2Cl$	44 (40.2, M^+), 43 (19.4, CH_3CO), 29 (100, CHO).

decompose in the presence of sodium bromide an additional product was 1-bromo-2-chloroethane.

Aqueous decomposition of the corresponding specifically deuterium labelled triazene 1-(p-cyanophenyl)-3-(2-chloro-1,1-dideuterioethyl)triazene 32 afforded acetaldehyde, 1,2,-dichloro-1,1-dideuterioethane, a mixture of the isomeric deuterium labelled 2-chloroethanols bearing the deuterium predominantly on the carbon bearing hydroxyl (92:8); a mixture of isomeric deuterium labelled N(2-hydroxyethyl)-p-cyanoanilines bearing deuteriums predominantly on the carbon adjacent to hydroxyl (70:30) together with other deuterated involatile products corresponding to those obtained from 16. Reaction of 32 in the presence of potassium bromide gave in addition labelled 1-bromo-2-chloroethane with the retention of both deuteriums most probably present on both carbons. When the isomeric deuterium labelled 1-(p-cyanophenyl)-3-(2-chloro-2,2-dideuterioethyl)triazene 33 was allowed to decompose in pH 7 phosphate buffer it gave acetaldehyde with deuterium in the formyl group, 1,2-dichloro-1,1-dideuterioethane, a mixture of the isomeric deuterium labelled 2-chloroethanols bearing deuterium predominantly on the carbon bearing chlorine, (92:8); a mixture of isomeric deuterated N-(2-chloroethyl)*p*-cyanoaniline bearing two deuteriums predominantly on the carbon adjacent to nitrogen (53:47); a mixture of isomeric deuterium labelled N-(2-hydroxyethyl)-p-cyanoanilines bearing two deuteriums predominantly on the carbon adjacent to nitrogen

(70:30) and deuterium labelled 1,2-di(p-cyanoanilino) ethane. Reaction of 33 in the presence of potassium bromide gave in addition labelled 1-bromo-2-chloroethane, again, with the retention of both deuteriums that are probably present on both carbons.

When 1-(p-cyanophenyl)-3-(2-chloro-2,2-dideuterioethyl) triazene 33 was allowed to react with 3,5-dinitrobenzoic acid the product was 2-chloro-2,2-dideuterioethyl 3,5-dinitrobenzoate 38 exclusively.

Aqueous decomposition of 1-(p-cyanophenyl)-3-(2-chloropropyl)triazene 34 at pH 7 and 37° afforded acetone; 2-chloro-1-propanol and 1-chloro-2-propanol (3:2); 1,2-dichloro-propane and 2-chloropropene as well as p-cyanoaniline.

Representative examples of two 2-fluoroethyl derivatives were allowed to decompose in buffered aqueous solution and their products identified and quantified. 5-[3-(2-fluoroethyl)-1-triazenyl]imidazole-4-carboxamide 35 afforded 2-fluoroethanol (79% of volatiles), acetaldehyde and 1,2-difluoroethane (together constituting 21% of volatiles), 5-aminoimidazole-4-carboxamide (90% of involatiles) and 5-(2-fluoroethylamino)imidazole-4-carboxamide (~1%).

Decomposition of 1-(p-cyanophenyl)-3-(2-fluoroethyl)-triazene 15 gave 2-fluoroethanol (76% of total volatiles), acetaldehyde and 1,2-difluoroethane (together constituting 24% of volatiles); p-cyanoaniline (86% of involatiles),

N-(2-hydroxyethyl)*p*-cyanoaniline (4% of involatiles) and a trace of N-(2-fluoroethyl)*p*-cyanoaniline. The relative stabilities of the triazenes as a function of structural variations as well as the pH of the medium were also established.

The kinetic half lives of the 2-haloalkyltriazenes in buffered aqueous solution could be conveniently determined electrochemically by following the disappearance of the polarographic wave characteristic of the triazene which appears in the range -0.910 to -1.038 V (Table 3). The observed half lives range from *ca.* 2 min to *ca.* 38 min. In the 1-(p-cyanophenyl)triazenes the sequence of stabilities depends on the halogen substituent i.e. F>Cl>Br. For a given halogen substituent in the 2-haloethyl-triazenes the sequence of stabilities depends on the nature of the p-aryl substituent: i.e. $CH_3CO>CH_3CH_2OCO>CN$. There was also observed to be a marked dependence of the decomposition half-life on the length of the alkyl chain bearing the halogen in the series of 1-(p-cyanophenyl)-3-(ω-chloroalkyl) triazenes such that butyl>propyl>pentyl>ethyl.

In the case of 1-(p-cyanophenyl)-3-(2-fluoroethyl)-trizene 15 a marked pH dependence on the rate of decomposition at 26° was observed (Table 4). The rate of decomposition increased rapidly as the pH was lowered in the range 8-6 such that $t_{\frac{1}{2}}$ at pH 8,7 and 6 were 10,080 sec, 7549 sec and 3175 sec respectively. The rate of the more rapid decomposition of 1-(p-cyanophenyl)-3-(2-chloroethyl)tri-

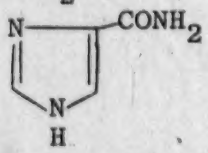
TABLE 3

HALF-LIFE ($t_{\frac{1}{2}}$) OF HALOALKYLARYLTRIAZENES DETERMINED POLAROGRAPHICALLY IN PHOSPHATE BUFFERED (0.01 M, pH 7.1) (95:5) AQUEOUS ACETONITRILE AT 37.5°

$pYC_6H_4NH-N=N-(CH_2)nX$

No Triazene	Y	X	n	Reduction potential (volts)	$t_{\frac{1}{2}}$ (sec)
15	CN	F	2	-0.925	1392
16	CN	Cl	2	-0.925	104
17	CN	Br	2	-0.942	94
18	CH_3CO	F	2	-0.925	2304
20	COOEt	F	2	-0.970	1891
21	COOEt	Cl	2	-0.980	164
34	CN	(2-Cl)	3	-0.930	1494
24	CN	Cl	3	-0.945	396
25	CN	Cl	4	-0.945	972

Table 3, continued

No Triazene	Y	X	n	Reduction potential (volts)	$t_{\frac{1}{2}}$ (sec)
26	CN	Cl	5	-0.910	234
27	CH_3CO	Cl	3	-0.935	264
20[+]	CO_2Et	F	2	-0.965	1522
35[+]		F	2	-1.038	704

[+]Determined in 95:5 aqueous DMSO.

TABLE 4

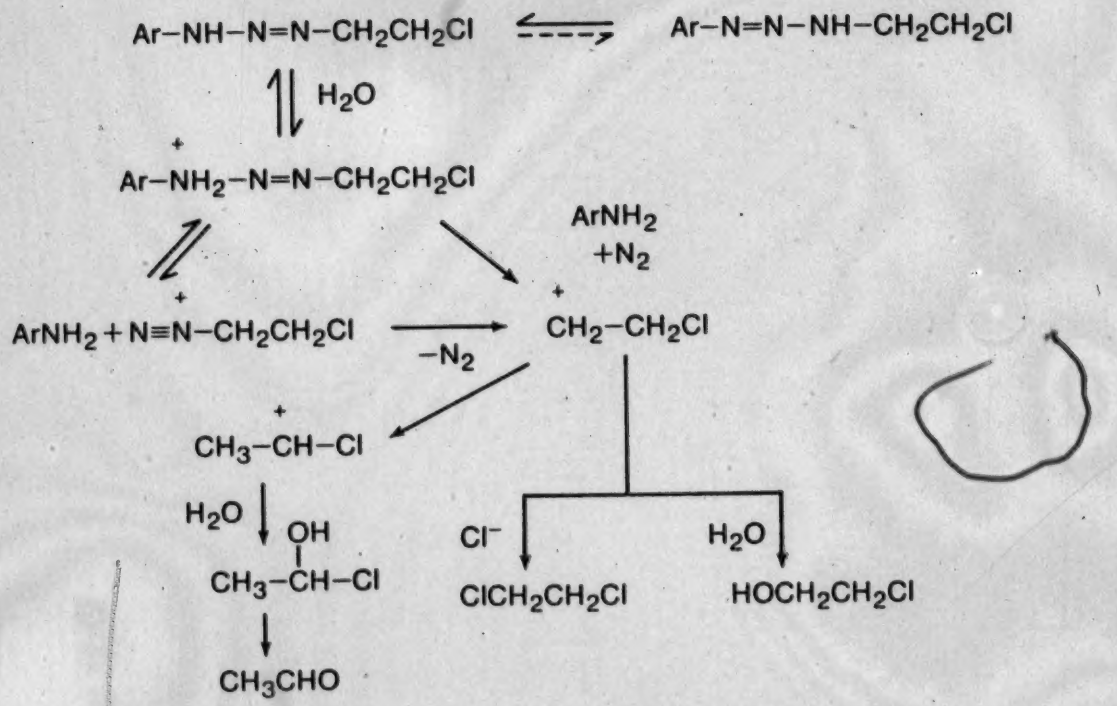
VARIATION OF HALF-LIFE ($t_{\frac{1}{2}}$) OF HALOALKYLARYLTRIAZENES POLAROGRAPHICALLY AS A FUNCTION OF SOLUTION pH AT 26°

Triazene	pH	$t_{\frac{1}{2}}$ (sec)
16	4.65	161
	5.95	355
	6.0	378
	6.95	420
	8.0	516
	9.3	718
15	6.0	3175
	7.0	7549
	8.0	10,080

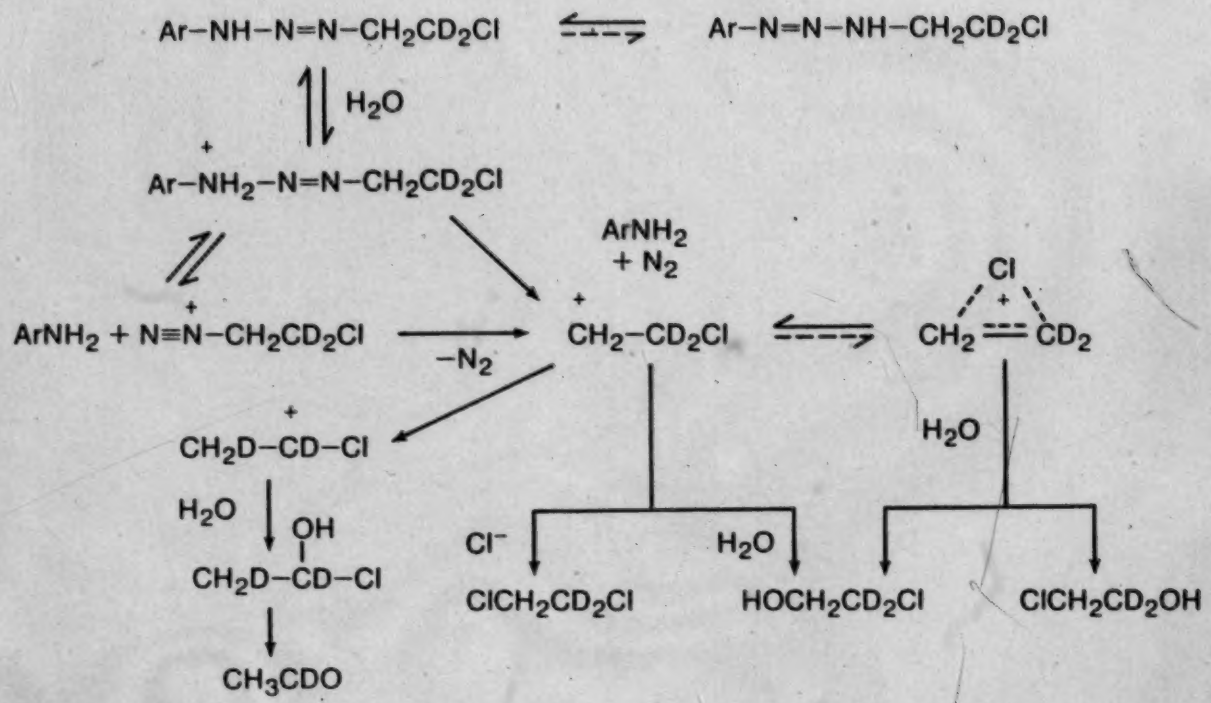
azene 16 at 26° also showed a marked and progressive increase on lowering the pH in the wider range of 9.3 to 4.65. The types of volatile products formed from aqueous decomposition of the 2-haloethylaryltriazenes are consistent with the general Scheme 4. The observed increase in the rate of decomposition of the triazenes with lowered pH suggests a protonation step on the major tautomer first. This is followed by cleavage to produce the observed aromatic amine and the 2-haloethyldiazonium ion or (its kinetic equivalent) the 2-haloethyl cation. Analogy with the reactions of 2-haloethylnitrosoureas suggests hydride transfer and hydrolysis of the latter can give rise to acetaldehyde.[54,55] Alternatively nucleophilic attack by halide ion or water on reactive species such as the diazonium ion, 2-haloethyl cation and/or the conjugate acid of the triazene gives rise to 1,2-dihaloethane and 2-haloethanol, respectively. The results with the specifically deuterated 2-haloethylaryltriazenes provided further insight into these pathways and the possible participation of the chloronium ion species.

The deuterium labelled products obtained from the 2,2-dideuterio-2-chloroethyltriazene may be interpreted in terms of Scheme 5. The retention of deuterium label in the formyl group of the acetaldehyde is consistent with generation of a transient 2-chloroethyl cation, a subsequent hydride shift to give the 1-chloroethyl cation and the hydrolysis of the latter. While either S_N2 nucleo-

Scheme 4


Ar–NH–N=N–CH2CH2Cl
Ar–N=N–NH–CH2CH2Cl
H2O
Ar–NH2–N=N–CH2CH2Cl
ArNH2
+N2
ArNH2 + N≡N–CH2CH2Cl
–N2
CH2–CH2Cl
CH3–CH–Cl
H2O
OH
CH3–CH–Cl
CH3CHO
Cl⁻
H2O
ClCH2CH2Cl
HOCH2CH2Cl

Scheme 5

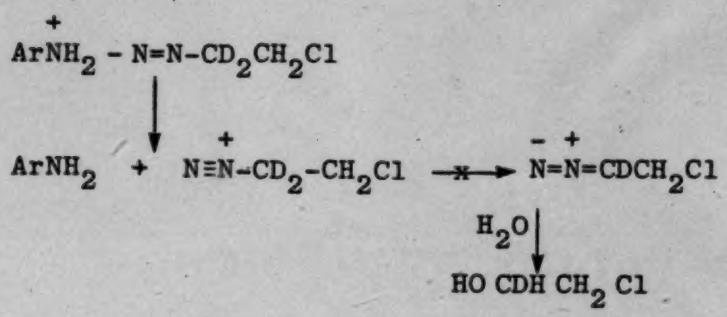

Ar–NH–N=N–CH2CD2Cl
Ar–N=N–NH–CH2CD2Cl
H2O
Ar–NH2–N=N–CH2CD2Cl
ArNH2 + N2
ArNH2 + N≡N–CH2CD2Cl
–N2
CH2–CD2Cl
Cl
CH2
CD2
H2O
CH2D–CD–Cl
H2O
OH
CH2D–CD–Cl
CH3CDO
Cl–
H2O
ClCH2CD2Cl
HOCH2CD2Cl
ClCH2CD2OH

philic attack by chloride ion on the conjugate acid of the triazene or S_N1 reaction via the 2-chloroethyl cation accounts for the 1,2-dichloroethane, the observed formation of a mixture of labelled 2-chloroethanols requires the intermediacy of another reactive species. The mixture of 2-chloroethanols contains predominantly (*ca.* 10:1) the isomer with deuterium adjacent to chlorine. As is the case with the corresponding 2-chloroethylnitrosoureas,[54] the small amount (5-10%) of deuterium scrambling is consistent with a small contribution of the chloronium ion species in the overall decomposition.

Entirely complementary results are obtained in the decomposition of the 1,1-dideuterio-2-chloroethyltriazene. In this case, and as predicted from a pathway corresponding to Scheme 5, only unlabelled acetaldehyde is obtained. In this case however, the two labelled 2-chloroethanols are obtained in a ratio of (10:1) containing predominantly the isomer with deuterium adjacent to hydroxyl, again in accord with a small contribution from the chloronium ion. Capture of the latter species by added bromide ion accounts for the formation of 1-bromo-2-chloroethane. Alternative decomposition pathways may be envisaged for the 2-haloethylaryltriazenes which would be indistinguishable from the main pathways outlined in Schemes 4 and 5 e.g. via the formation of a diazohydroxide.

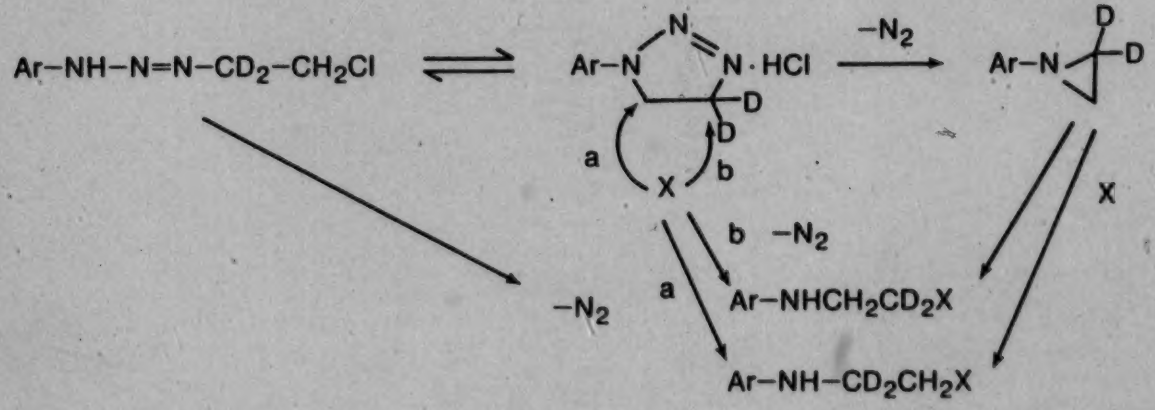
$$Ar\text{-}NH\text{-}N{=}NCH_2CH_2Cl \xrightarrow{H_3O^+} ArNH_3^+ + HO\text{-}N{=}N\text{-}CH_2CH_2Cl$$

The deuterium labelling experiments do however preclude the intermediacy of a diazoalkane since this requires the loss of one deuterium from the 2-chloroethanol and 1,2-dichloroethane isolated from the 1,1-dideuterio-2-chloroethyltriazene.



Four involatile products were identified and quantitated from aqueous decomposition of triazene 16. The use of specific deuterium labelling was especially informative in determining the origin of these products, while there are precedents for the direct loss of nitrogen from monomethyltriazenes.[47] A similar loss of nitrogen from 32 could yield 2-chloroethyl-p-cyanoaniline. However, the observation of deuterium scrambling favouring the rearrangement product (52:48) demands the intermediacy of a cyclic intermediate, but not a symmetrical one. The facts are in accord with the intermediate formation of the aryl 1,2,3-triazolinium salt (for which the precedents exist[49-51]), as shown in Scheme 6. The much more marked preference for the formation of the rearrangement product with the 2-hydroxyethyl-p-cyanoaniline (70:30) confirms an unsymmetrical cyclic intermediate with in this case preferential

Scheme 6



X = $\bar{C}l$, $\bar{O}H$ or $pCNC_6H_4NH_2$

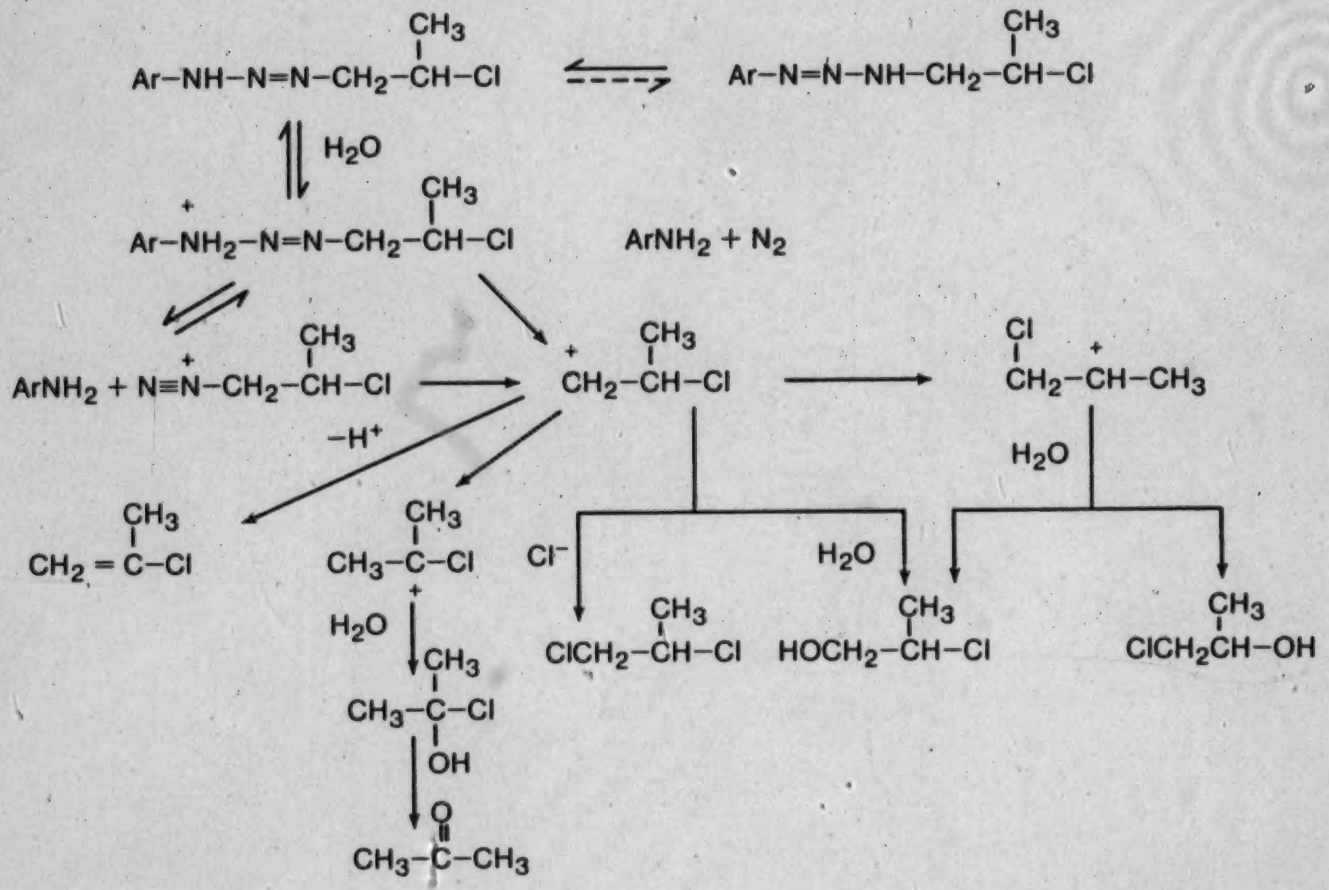
attack by water at position 4 of the aryl 1,2,3-triazoline. Whereas the aziridine may participate as an intermediate it alone cannot explain the observed results. Attack by p-Cyanoaniline on the intermediate aryltriazolinium salt (or aziridine) accounts for the formation of 1,2-p-cyanoanilinoethane. The results with the isomeric 1-(p-cyanophenyl)-3-(2-chloro-2,2-dideuterioethyl)triazene 33 are entirely consistent with Scheme 6.

The effects of a substituent alpha to the halogen were observed in the decomposition of 1-(p-cyanophenyl)-3-(2-chloropropyl)triazene 34. The formation of acetone is rationalized by initial formation of the diazonium ion (from protonation of the preferred tautomer) and then a formation of the 2-chloropropyl-3-cation leads to acetone by the steps indicated in Scheme 7. This requires a hydride transfer from the 2-chloropropyl cation to form the more stable 2-chloropropyl-2-cation.

In this case proton loss from the initially formed cation to give 2-chloropropene is favored by the additional methyl substituent in contrast to the 2-chloroethylaryl-triazene case where no detectable quantities of vinyl chloride were produced (Schemes 4 and 5). (See chapter IV for further discussion on this issue).

Nucleophilic attack by chloride ion (either S_N2 on the conjugate acid of the triazene or the diazonium ion or by S_N1 reaction via the 2-chloropropyl-1-cation) accounts for the formation of 1,2-dichloropropane. Since both possible

Scheme 7


Ar–NH–N=N–CH2–CH(CH3)–Cl
Ar–N=N–NH–CH2–CH(CH3)–Cl
H2O
Ar–NH2–N=N–CH2–CH(CH3)–Cl
ArNH2 + N2
ArNH2 + N≡N–CH2–CH(CH3)–Cl
CH2–CH(CH3)–Cl
CH2–CH(Cl)–CH3
–H+
H2O
CH2=C(CH3)–Cl
CH3–C(CH3)–Cl
Cl–
H2O
ClCH2–CH(CH3)–Cl
HOCH2–CH(CH3)–Cl
ClCH2CH(CH3)–OH
H2O
CH3–C(CH3)(OH)–Cl
CH3–CO–CH3

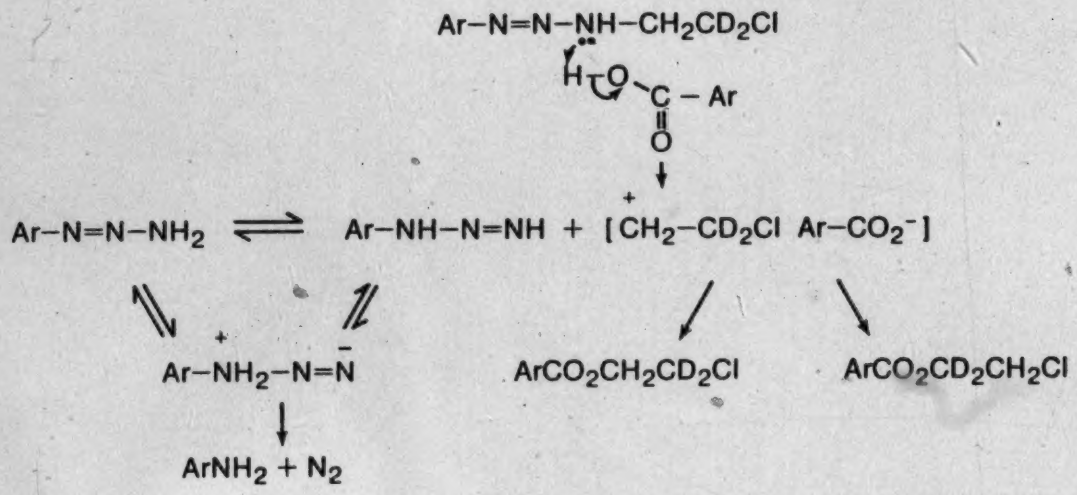
isomeric chloropropanols were identified as products this requires a rearrangement of the intermediate 2-chloro-1-propyl cation to the more stable 1-chloro-2-propyl cation. Thus in this example we employ a methyl group as a label rather than deuterium in Scheme 7. The observed ratio of 3:2 of 2-chloro-1-propanol:1-chloro-2-propanol is in accord with the expected greater contribution of this pathway compared with the approximately 10% contribution of the chloronium ion[56,57] in Scheme 5. The fact that a greater yield of the chloropropanol product of the less stable cation is obtained suggests a substantial contribution of direct S_N2 attack on the precursor diazonium ion or the conjugate acid of the triazene itself (Scheme 7).

The observed order of decomposition of the homologous series of ω-haloalkyl-(p-cyanophenyl)triazenes 16>26>24>25 *i.e.* chloroethyl>chloropentyl>chloropropyl>chlorobutyl deserves a comment. The fact that the chloroethyl homologue decomposes fastest is in accord with the intermediacy in this case only of the triazoline species. Analogous cyclization for 24, 25, and 26 is presumably precluded for entropic reasons. Moreover, only in the case of 16 can the chloronium ion contribute. The intervention of other pathways may be envisaged involving 1,2-hydride shift leading to a number of possible cationic intermediates in the 3, 4 and 5 carbon homologues. This may account for the observed rate sequences which requires detailed individual examination.

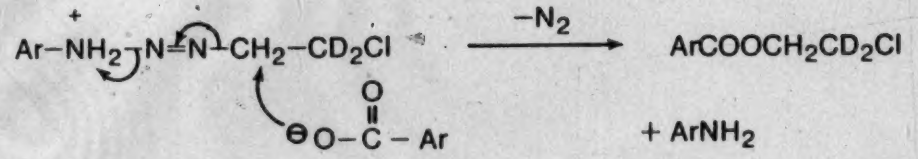
In connection with the chloronium ion species, the observation of deuterium label scrambling in the decomposition of the 2-chloroethyl triazenes afforded an opportunity to investigate the mechanism of alkylation by these triazenes. Such a reaction has direct bearing on the cytotoxicity of triazenes which has been attributed in part to the alkylation of biological macromolecules. Alkylation of 3,5-dinitrobenzoic acid with 1-(p-cyanophenyl)-3-(2-chloro-2,2-dideuterioethyl)triazene 33 afforded only the 2-chloro-2,2-dideuterioethyl 3,5-dinitrobenzoate 38 *i.e.* no detected label scrambling had occurred. This result is clearly at variance with one mechanism that has been suggested for this reaction (Scheme 8).[58] This mechanism requires the formation of a solvent caged ion-pair which, since it permits Wagner-Meerwein rearrangement of the intermediate cation, would predict a certain degree of label scrambling as shown. A more likely pathway is by S_N2 displacement which correctly predicts the lack of label scrambling which is observed (Scheme 9). This result is then in accord with the previous conclusion of a substantial contribution from S_N2 mechanisms in the formation of products from the intermediate diazonium ions or the conjugate acids of the triazenes in Schemes 4, 5 and 7. However, this issue has been taken up for further studies and comments in Chapter IV.

The above findings on the synthesis, the identification of aqueous decomposition products and details of the decom-

Scheme 8



Scheme 9



position pathways of new 2-haloethylaryltriazenes has assisted in the interpretation of their anti-leukemic action as discussed in a subsequent chapter.

EXPERIMENTAL

Throughout this work melting points were determined on a Fisher-Johns apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Grating Infrared Spectrophotometer Model 421 and generally only the principal peaks are reported. Absorption spectra were measured in either distilled water or "spectro-grade" solvents and were run on either a Beckman DB Spectrophotometer, a Gilford 250 Spectrophotometer, or a Unicam SP1700 UV Spectrophotometer. Proton magnetic resonance spectra were recorded on Varian HA-100 or Bruker WH-200 instruments and were generally measured on a 10-15% (w/v) solutions of the compound in the appropriate deuterated solvent. The reference compound was tetramethylsilane (TMS). Line positions are reported in ppm from the reference. Mass spectra were determined with Associated Electrical Industries MS2, MS9, MS12, or MS50 (DS50) mass spectrometers. In general the ionization energy was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 1500. Kieselgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin layer chromatography. Microanalyses were carried out by Mrs. D. Mahlow of this department.

1(3)-Aryl-3(1)-2-Haloalkyltriazenes

General Procedure

A solution of the aromatic amine (10 mmol) in concentrated hydrochloric acid (2.5 ml), diluted with water (12 ml) was diazotized at 0°C with sodium nitrite (0.80 g), dissolved in the minimum amount of water and the resulting solution was stirred for 30 min or until clear. Excess acid was neutralized by addition of calcium carbonate (1.6 g). 2-Haloalkylamine hydrohalide (10 mmol) was dissolved in 5 ml water and deprotonated with potassium bicarbonate (11 mmol) (added solid) by stirring at 0°C until effervescence stopped. The diazonium salt solution was filtered cold and quickly transferred to the flask containing 2-haloalkylamine free base and then the reaction mixture was stirred for 5-10 min at 0°. A precipitate of the triazene usually appeared immediately and was washed with cold water, air dried for 1 min and transferred to a precooled test tube. The product was dissolved in a minimum amount of methylene chloride at room temperature whereupon an aqueous layer separated. The methylene chloride layer was carefully pipetted out and transferred to another precooled container. The triazene was precipitated out by cooling the methylene chloride solution to -30° and adding cold petroleum ether dropwise. The precipitate was filtered, washed with cold petroleum ether, and air dried for 1-2 min.

Special precaution was exercised during the condensation of 2-bromoethylamine with the diazonium salt as well as in handling the product. The condensation was usually carried out at -10° and the precipitate was transferred to a precooled test tube immediately after filtration in order to avoid decomposition. 2-Haloalkyltriazenes can be stored in dry-ice for an indefinite period. The general procedure afforded the following triazenes.

1-(p-Nitrophenyl)-3-(2-fluoroethyl)triazene 12

(2.0 g 95%), mp 98-100°; ν_{max} 3184, 3142, 1608, 1595, 1515, 1340 cm^{-1}; δ (CD_2Cl_2, -20°C) 4.06(2t, J_H = 5 Hz, J_F = 30 Hz, 2H, N-CH_2), 4.81 (2t, J_H = 5 Hz, J_F = 47 Hz, 2H, CH_2-F), 7.19-8.36 (m, 4H, aromatic); 8.5 and 9.55 (br 2s, exchangeable, 1H, NH) M^+ 212.0715 (45.90%) (calcd. for $C_8H_9FN_4O_2$, 212.0710), m/e 184.0655 (M-N_2, calcd. for $C_8H_9FN_2O_2$, 184.0648), 164.0589 (2.38%) (M-N_2-HF, calcd. for $C_8H_8N_2O_2$, 164.0586) 150.0305 (68.78%) (M-NH-CH_2CH_2F, calcd. for $C_6H_4N_3O_2$, 150.0303), 138.0431 (27%) (M-N_2CHCH_2F, calcd. for $C_6H_6N_2O_2$, 138.0415), 122.0246 (100%) (calcd. for $C_6H_4NO_2$, 122.0228).

1-(*p*-Nitrophenyl)-3-(2-chloroethyl)triazene 13

(1.8 g, 79%) (mp 64-66°C), ν_{max} 3180, 3140, 1605 and 1595 cm^{-1}. δ ($CDCl_3$, -20°) 3.9 (t, 2H, CH_2-Cl), 4.1 (t, 2H, N-CH_2), 7.2 - 8.35 (m, 4H, aromatic), 8.5 and 9.6 (1H, exchangeable, N-H); M^+ 228.0420 (2.45%) (calcd. for

$C_8H_9ClN_4O_2$, 228.0414), m/e 200.0361 (23.02%) ($M-N_2$, calcd. for $C_8H_9ClN_2O_2$, 200.0353), 164.0588 (18.12%) ($(M-N_2)-HCl$, calcd. for $C_8H_8N_2O_2$, 164.0586) 150.0346 (4.12%) ($M-NHCH_2CH_2Cl$, calcd. for $C_6H_4N_3O_2$, 150.0304), 138.0434 (37.79%) ($M-N_2CHCH_2Cl$, calcd. for $C_6H_6N_2O_2$, 138.0429).

1-(*p*-Nitrophenyl)-3-(2-bromoethyl)triazene 14

(1.2 g, 44%), mp 30-31°, ν_{max} (due to rapid thermal decomposition the absorptions were too broad to be assigned to definite frequencies); δ ($CDCl_3$, -50°C) 3.8 (t, 2H, CH_2-Br), 4.25 (t, 2H, N-CH_2), 7.2 - 8.3 (m, 4H, aromatic), 8.7 and 9.7 (br, 2s, 1H, N-H), m/e 164.0599 (1.25%) ($(M-N_2)-HBr$, calcd. for $C_8H_8N_2O_2$, 164.0586), 138.0434 (100%).

1-(*p*-Cyanophenyl)-3-(2-fluoroethyl)triazene 15

(1.8 g, 93%), mp 95-96°; ν_{max} 3183, 3161, 2228 and 1609. δ ($CDCl_3$, -20°) 4.08 (2t, J_H = 5 Hz, J_F = 30, 2H, N-CH_2), 4.8 (2t, J_H = 5 Hz, J_F = 47, 2H, CH_2-F), 7.2 - 7.75 (m, 4H, aromatic) 8.5 and 9.55 (br, 2s, exchangeable, 1H, N-H), M^+ 192.0804 (26.09%) (calcd. for $C_9H_9FN_4$, 192.0811), m/e 164.0744 (1.39%) ($M-N_2$, calcd. for $C_9H_9FN_2$, 164.0750), 144.0683 (4.02%) ($(M-N_2)-HF$, calcd. for $C_9H_8N_2$, 144.0688) 130.0403 (33.87%) ($M-NHCH_2CH_2F$, calcd. for $C_7H_4N_3$, 130.0405), 118.0528 (32.11%) ($M-N_2-CH_2CH_2F$, calcd. for $C_7H_6N_2$, 118.0531), 102.0342 (100%).

1-(*p*-Cyanophenyl)-3-(2-chloroethyl)triazene 16

(1.0 g, 48%), mp 68°, ν_{max} 3181, 3159, 2228 and 1608 cm^{-1}. δ (CD_2Cl_2, -45°) 3.9 (t, 2H, CH_2-Cl), 4.1 (t, 2H, N-CH_2), 7.2 - 7.75 (m, 4H, aromatic) 8.55 and 9.6 (br, 2s, 1H, exchangeable, N-H); m/e 182.0424 (7.23%) (M-N_2, calcd. for $C_9H_9ClN_2$, 182.0424), 144.0682 (9.74%) ((M-N_2)-HCl, calcd. for $C_9H_8N_2$ 144.0688), 132.0638 (11.06%), 131.0638 (100%), 118.0530 (62.79%).

1-(*p*-Cyanophenyl)-3-(2-bromoethyl)triazene 17

(Yield 10-40%) mp 39°. Compound (17) decomposed during ir scan. δ (CD_2Cl_2, -45°), 3.7 (t, 2H, CH_2Cl), 4.9 (t, 2H, N-CH_2), 7.2 - 7.8 (m, 4H, aromatic), 8.7 (br, 1s, integrated for less than one proton, normally observed second broad singlet could not be traced, N-H), m/e 225.9925 (20.26%) (M-N_2, calcd. for $C_9H_9N_2Br$ 225.9929), 144.0675 (9.74%) (M-N_2) -HBr calcd. for $C_9H_8N_2$, 144.0688), 132.0638 (25.25%), 118.0531 (26.52%).

1-(*p*-Acetylphenyl)-3-(2-fluoroethyl)triazene 18

(0.8 g, 38%) mp 92-93°, ν_{max} 3192, 3161 and 1600 cm^{-1}; δ (CD_2Cl_2, -45°) 2.6 (s, 3H, Me), 4.08 (2t, J_H = 5 Hz, J_F = 30 Hz, 2H, N-CH_2), 4.84 (2t, J_H = 5 Hz, J_F = 47 Hz), 7.15 - 8.15 (m, 4H, aromatic), 8.94 and 10.0 (br, 2s, N-H); M^+ 209.0964 (35.12%) (calcd. for $C_{10}H_{12}FN_3O$, 209.0964), 182.0952 (.41%) (M-N_2, calcd. for $C_{10}H_{13}FNO$, 182.0981),

148.0734 (16.55%), 135.0682 (44.49%), 120.0460 (87.20%) 119.0490 (100%).

1-(*p*-Acetylphenyl)-3-(2-chloroethyl)triazene 19

(0.6 g, 27%), mp 48-49°, ν_{max} 3188, 3158 and 1600 cm^{-1}. δ (CD_2Cl_2, -45°) 2.62 (s, 3H, Me), 3.9 (t, 2H, CH_2Cl), 4.12 (t, 2H, N-CH_2), 7.15 - 8.1 (m, 4H, aromatic), 8.7 and 9.75 (br, 2s, N-H); m/e 197.0607 (21.67%) (M-N_2, calcd. for $C_{10}H_{12}ClNO$, 197.0607), 161.0851 (2.01%) ((M-N_2)-HCl, calcd. for $C_{10}H_{11}NO$ 161.0841), 148.0760 (77.08%), 120.0450 (100%).

1-(*p*-Ethoxycarbonylphenyl)-3-(2-fluoroethyl)triazene 20

(1.6 g, 56%), mp 85-87°, ν_{max} 3194, 3174, 1714, 1610: δ (CD_2Cl_2, 200 MHz, -55°), 1.35 (t, 3H, Me), 4 (2t each accompanied by a multiplet at the base, J_H = 5 Hz, J_F = 47 Hz, 2H, N-CH_2) 4.27 (q, 2H, O-CH_2), 4.78 (2t, each accompanied by a triplet at the base, J = 5 Hz, J_F = 47 Hz, 2H, CH_2-F), 7.1 - 8.05 (m, 4H, aromatic), 8.6 (t, N-H, tautomer), 9.85 (br, s, N-H, tautomer). M^+ 239.1077 (49.67%) (calcd. for $C_{11}H_{14}FN_3O_2$, 239.1070), m/e 211.1017 (4.78%) (M-N_2, calcd. for $C_{11}H_{14}FNO_2$, 211.1008), 194.0737 (12.58%) (M-CH_2CH_3, calcd. for $C_9H_9FN_3O_2$, 194.0730), 191.0953 (6.93%) ((M-N_2-HCl, calcd. for $C_{11}H_{13}NO_2$ 191.0947), 165.0795 (64.65%), 149.0599 (100%).

1-(*p*-Ethoxycarbonylphenyl)-3-(2-chloroethyl)triazene 21

(1.8 g, 70%) mp 58-60°, ν_{max} 3193, 3171, 1715 and 1605 cm^{-1}. δ ($CDCl_3$, -30°), 1.38 (t, 3H, Me), 3.84 (t, 2H, CH_2-Cl), 4.06 (t, 2H, N-CH_2), 4.34 (q, 2H, O-CH_2), 7.1 - 8.1 (m, 4H, aromatic), 8.55 and 9.58 (br, 2s, N-H). m/e 227.0716 (21.16%) (M-N_2, calcd. for $C_{11}H_{14}ClNO_2$, 227.0713), 191.0949 (19.85%), ((M-N_2)-HCl, calcd. for $C_{11}H_{13}NO_2$, 191.0446), 165.0793 (71.29%), 120.0449 (100%).

1-(*o*-Nitrophenyl)-3-(2-fluoroethyl)triazene 22

(1.5 g; 70%), mp 30-31°, ν_{max} 3322, 1613, 1515, 1338; δ ($CDCl_3$, -20°), 4.08 (2t, J_H = 5 Hz, J_F = 30 Hz, 2H, N-CH_2), 4.84 (2t, J_H = 5 Hz, J_F = 47 Hz, 2H, CH_2-F), 6.94 - 8.34 (m, 4H, aromatic), 11.74 (s, 1H, NH); M^+ 212.0712 (67.61) (calcd. for $C_8H_9FN_4O$, 212.0710), m/e 184.0650 (2.18%) (M-N_2, calcd. for $C_8H_9FN_2O_2$, 184.0648), 164.0589 (1.78%) ((M-N_2)-HF, calcd. for $C_8H_8N_2O_2$, 164.0586), 138.0430 (100%).

1(3)-(*o*-Nitrophenyl)3(1)-(2-chloroethyl)triazene 23

(1.8 g, 79%) mp 55°, ν_{max} 3329, 1610, 1513, 1338; δ ($CDCl_3$, -20°), 3.92 (t, 2H, CH_2-Cl), 4.12 (t, 2H, N-CH_2), 6.94 - 8.34 (m, 4H, aromatic), 11.76 (s, 1H, NH), M^+ 228.0414 (21.74%) (calcd. for $C_6H_9ClN_4O_2$, 228.0414), m/e 200.0353 (0.76%) (M-N_2, calcd. for $C_8H_9ClN_2O_2$, 200.0353), 164.0592 (1.02%) (calcd. $C_8H_8N_2O_2$, 164.0586), 138.0429 (44.07%), 63.0121 (100%) (calcd. for C_2H_4Cl, 63.0001).

The following triazenes were also prepared by the general procedure. The diazonium salt solutions were allowed to react with the corresponding chloro-alkylamines and the products were purified by recrystallization at low temperature.

1-(*p*-Cyanophenyl)-3-(3-chloropropyl)triazene 24

Mp 64-65° (1.5 g, 67%) ν_{max} 3186, 3162, 2228 and 1610 cm^{-1}. δ (CD_2Cl_2, -15°) 2.18 (t, 2H, $-CH_2-$), 3.65 (t, 2H, CH_2-Cl), 6.85 (t, 2H, $N-CH_2$) 7.2 - 7.8 (m, 4H, aromatic), 8.5 and 9.5 (br, 2s, exchangeable, N-H) M^+ 222.0673 (7.79%) (calcd. for $C_{10}H_{11}ClN_4$. 222.0673), 194.0609 (3.62%) ($M-N_2$ calcd. for $C_{10}H_{11}ClN_2$, 194.0611), 158.0841 (7.93%) ($(M-N_2)-HCl$, calcd. for $C_{10}H_{10}N_2$, 158.0843), 132.0639 (2.55%), 131.0438 (1.26%), 118.0531 (100%).

1-*p*-Cyanophenyl)-3-(4-chlorobutyl)triazene 25

(1.6 g 67%) mp 82°, ν_{max} 3186, 3163, 2220, 1608 cm^{-1}. δ (CD_2Cl_2, -15°) 1.85 (br, m, 4H, $-CH_2CH_2-$), 3.4 - 3.85 (br, m, 4H, $N-CH_2$ and CH_2-Cl), (7.2 - 7.8) (m, 4H, aromatic), 8.35 and 9.4 (br, 2s, exchangeable, N-H); M^+ 236.0829 (12.05%) (calcd. for $C_{11}H_{13}ClN_4$, 236.0829), m/e 208.0768 (1.53%) ($M-N_2$, calcd. for $C_{11}H_{13}ClN_2$, 208.0768), 172.0921 (0.24%) ($(M-N_2)-HCl$, calcd. for $C_{11}H_{12}N_2$, 172.1010) 131.0573 (16.39%), 118.0529 (56.77%), 102.0343 (100%).

1-(*p*-Cyanophenyl)-3-(5-chloropentyl)triazene 26

(2.16 g, 84%), mp 66°, ν_{max} 3183, 3160, 2220 and 1610 cm^{-1}. δ (CD_2Cl_2, -15°) 1.7 (br, m, 6H), 3.65 (br, sextet, N-CH_2 and CH_2-Cl), 7.2 - 7.8 (m, 4H, aromatic), 8.4 and 9.5 (br, 2s, exchangeable, N-H), M^+ 250 (6.12%) calcd. for $C_{12}H_{15}ClN_4$, 250.0985), m/e 222.0926 (1.33%) (M-N_2, calcd. for $C_{12}H_{15}N_2$, 222.0924), 131.0569 (12.78%), 118.0529 (100%).

1-(*p*-Acetylphenyl)-3-(3-chloropropyl)triazene 27

(1.2 g, 50%), mp 50-51°, ν_{max} 3186, 3157, 1674 and 1600 cm^{-1}. δ (CD_2Cl_2, -45°) 2.2 (p, 2H, -CH_2-), 2.62 (s, 3H, Me) 3.67 (t, 2H, CH_2-Cl), 3.92 (t, 2H, N-CH_2), 7.15 - 8.1 (m, 4H, aromatic), 8.5 and 9.65 (br, 2s, N-H), M^+ 239.0825 (1.70%) (calcd. for $C_{11}H_{14}ClN_3O$, 239.0826), 211.0765 (1.09%), (M-N_2, calcd. for $C_{11}H_{14}ClNO$, 211.0766), 175.0996 (3.56%) ((M-N_2)-HCl, calcd. for $C_{11}H_{13}NO$, 175.0997), 120.0450 (83.85%).

2-Amino-2,2-dideuterioethanol 28

This compound was prepared by reducing glycolonitrile with lithium aluminum deuteride following the procedure of Brundrett *et al.*[54] bp 40-41°C, ~0.15 mm (lit[54] bp 85-90°, ~10 mm).

2-Chloro-1,1-dideuterioethylaminehydrochloride 29

A solution of 28 (2 g, 21.6 mmol) in $CHCl_3$ (10 ml) was saturated with hydrogen chloride gas at 0°C. To the solution was added thionyl chloride (10 ml) diluted with $CHCl_3$ (10 ml) at 0° and the solution was allowed to warm slowly to 70°. The stirring was continued for 5 hr. The solvent and the excess of the thionyl chloride were removed under vacuum. The residue was purified by recrystallization from absolute ethanol. Yield 1.5 g, 60%, mp 141-143 (undeuterated, 143-146°).

2-Amino-1,1-dideuterioethanol 30

Compound 30 was prepared by reducing glycine ethyl ester with lithium aluminum deuteride following the procedure of Brundrett *et al.*[54] bp 35-38° ~0.15 mm, lit.[54] bp 83-88°C ~10 mm).

1-Chloro-1,1-dideuterioethylaminehydrochloride 31

The aminoethanol 30 was chlorinated following the procedure described for the chlorination of 28, mp 144-146°.

The following triazenes were also prepared by the general procedure. The diazonium salt solutions were allowed to react with the corresponding chloro-alkylamines and the products were purified by recrystallization at low temperature.

1-(*p*-Cyanophenyl)-3-(2-chloro-1,1-dideuterioethyl)-triazene 32

Mp 66-67°, ν_{max} 3181, 3160, 2224 and 1608 cm^{-1}. δ (CD_2Cl_2, -10°) 3.84 (s, 2H, CH_2-Cl), 7.2 - 7.75 (m, 4H, aromatic), 8.56 and 9.15 (br, 2s, exchangeable, N-H); M^+ 210.0644 (3.03%) (calcd. for $C_9H_7D_2ClN_4$, 210.0641), 182.0578 (18.69%) (M-N_2, calcd. for $C_9H_7D_2ClN_2$, 182.0580), 146.0810 (23.62%) ((M-N_2)-HCl, calcd. for $C_9H_6D_2N_2$, 146.0813), 118.0532 (100%).

1-(*p*-Cyanophenyl)-(3)-(2-chloro-2,2-dideuterioethyl)-triazene 33

Mp (67-68°), ν_{max} 3181, 3159, 2224 and 1608 cm^{-1}. δ (CD_2Cl_2, -15°) 4.03 (s, 2H, N-CH_2), 7.2 - 7.70 (m, aromatic), 8.5 and 9.45 (br, s, exchangeable, N-H); M^+ 210.0661 (0.50%) (calcd. for $C_9H_7D_2ClN_4$ 210.0641), m/e 182.0581 (63.95%) (M-N_2, calcd. for $C_9H_7D_2ClN_2$ 182.0580), 146.0811 (33.91%) ((M-N_2)-HCl, calcd. for $C_9H_6D_2N_2$, 146.0813), 133.0724 (100%).

1-(*p*-Cyanophenyl)-3-(2-chloropropyl)triazene 34

(1.5 g, 72%) mp 74-75°, compound 34 decomposed during ir scan. δ (CD_2Cl_2, -20°) 1.56 (2d, 3H, CH_3), 3.95 (2d, 2H, N-CH_2), 4.4 (m, 1H, CHCl), 7.2 - 7.8 (m, 4H, aromatic), 8.6 and 9.5 (br, 2s, exchangeable, N-H), M^+ 222.0672 (12.10%) (calcd. for $C_{10}H_{11}ClN_4$, 222.0672), 194.0613 (0.57%)

($M-N_2$, calcd. for $C_{10}H_{11}ClN_2$, 194.0611), 158.0844 (1.55%) (($M-N_2$)-HCl calcd. for $C_{10}H_{10}N_2$, 158.0844) 118.0530 (30.08%), 102.0344 (100%).

5-[3-(2-Fluoroethyl)-1-triazenyl]-imidazole-4-carboxamide 35

Compound 35 was prepared following the procedure of Shealy *et al.*[46] 2-Fluoroethylamine free base (400 mg, 6.3 mmol) was taken in dry ethyl acetate (30 ml) and stirred under nitrogen. To the stirred solution was added thoroughly dried 5-diazo-imidazole-4-carboxamide (800 mg, 4.6 mmol) in one portion. The reaction mixture was protected from light and the stirring was continued for 1 hr. The resulting white solid was collected by filtration, washed with ethyl acetate and dried *in vacuo* at room temperature, yielding 850 mg (92%) of the triazene mp 119°C (dec). ν_{max} 3478, 3249, 3079, 1645, 1585, 1430 and 1380 cm^{-1}, m/e 138.0396 (11.75%) ($M-NHCH_2CH_2F$, calcd. for $C_4H_4N_5O$, 138.0398), 137.0341 (100%). Anal. calcd. for $C_6H_9N_6O$: C, 36.0, H, 4.5; N, 42.0. Found: C, 35.6; H, 4.5, N, 41.6.

General Procedure for the Esterification of 3,5-Dinitrobenzoic Acid by Phenyl-2-haloalkyltriazenes

The triazene (4 mmol) was suspended in ether (15 ml) at -20°. To the stirring suspension was added dropwise 3,4-dinitrobenzoic acid (700 mg, 3.3 mmol) dissolved in ether (20 ml). After the addition was completed, the reac-

tion mixture was allowed to warm up to the room temperature and the stirring was continued for another 2 hr. The reaction mixture was filtered and the solvent was evaporated leaving a syrupy residue which on tituration with chloroform deposited most of the unreacted acid. The ester was purified by chromatography on silica using benzene as eluant. When the imidazole triazene 35 was used, the reaction mixture was stirred for two days at room temperature protected from light at all time. This procedure afforded the following esters in varying yield.

2-Fluoroethyl-3,5-dinitrobenzoate 36

From 15 and 35 yield 60% and 30% respectively, mp 84-85° (lit 86°). ν_{max} 1735, 1625, 1545 and 1340 cm^{-1} (in CH_2Cl_2). δ ($CDCl_3$, 200 MHz), 4.7 (m, 2H, O-CH_2), 4.99 (2m, 2H, CH_2-F), 9.18 - 9.3 (m, 3H, aromatic). Compound 36 was identical in every respect with a sample obtained from reaction of 3.5-dinitrobenzoyl chloride with 2-fluoroethanol.

2-Chloroethyl-3,5-dinitrobenzoate 37

From 16 yield 40%, mp 89-90° (lit 92°) ν_{max} 1735, 1625, 1545 and 1340 cm^{-1}. δ (CD_2Cl_2) 3.88 (t, 2H, CH_2-Cl) 4.72 (t, 2H, OCH_2), 9.14 - 9.28 (m, 3H, aromatic), M^+ 273.9981 (2.98%) (calcd. for $C_9H_7N_2O_6Cl$, 273.9992), m/e 239.0299 (5.78%) (M-Cl, calcd. for $C_9H_7N_2O_6$, 239.0304). Compound 37 was identical with a sample obtained from reaction of 3.5-dinitrobenzoylchloride with 2-chloroethanol.

2-Chloro-2,2-dideuterioethyl-3,5-dinitrobenzoate 38

From 33 yield 40%, mp 89°. ν_{max} 1735, 1625, 1535 and 1340 cm^{-1}. δ ($CDCl_3$) 4.6 (s, 2H, O-CH_2), 9.1 - 9.3 (m, 3H, aromatic); M^+ 276.0117 (8.53%) (calcd. for $C_9H_5D_2ClN_2O_6$, 276.0117). Anal. calcd. for $C_9H_5D_2ClN_2O_6$: C, 39.05%, N, 10.12%, Found: C, 39.18%; N, 9.83%.

2-Bromoethyl-3,5-dinitrobenzoate 39

From 17 yield 10% mp 82° (lit 85-86°) ν_{max} 1740, 1625, 1540 and 1340 cm^{-1}. δ ($CDCl_3$) 3.70 (t, 2H, CH_2-Br), 4.66 (t, 2H, O-CH_2), 9.1 - 9.3 (m, 3H, aromatic). Compound 39 was identical with a sample obtained from reaction of 3,5-dinitrobenzoylchloride with 2-bromoethanol.

METHODS

Polarographic Determination of Decomposition Rates for Triazenes

The Princeton Applied Research (PAR) Model 174A polarograph and 9300-9301 polarographic cell were used in a three electrode configuration which included an aqueous saturated calomel reference electrode (SCE), to which all potentials in this thesis are relative, a platinum counter electrode, and a dropping mercury electrode (DME) with a controlled 2s drop time. The temperature in the cell was maintained at 37.5 ± 0.2°C by circulation of thermostatted

water unless otherwise indicated. The resulting curves were recorded on a Houston 2000 X-Y recorder. The sample solutions were buffered at pH 7.1 with 0.01 M potassium phosphate buffer in 0.01 M KCl supporting electrolyte. The pH value of the sample solutions were measured with an Accumet Model 520 pH meter before each run.

Triazenes in general showed low solubility in aqueous solution, therefore 5% acetonitrile aqueous solution was used. For triazenes differential pulse polarography of the aqueous solution was sufficiently sensitive and was used in each case. All solutions were deareated with purified nitrogen for 2 min before a run and blanketed with it during the run.

Studies Related to the Aqueous Decomposition of 2-Haloethyltriazenes

(a) Identification of Volatile Products

GC analyses were performed on a Hewlett-Packard Model 5840 A gas chromatograph equipped with flame ionization detector. GC MS analyses were performed on an AEI MS-12 spectrometer using helium gas flow rate of 22 ml/min. Samples were injected onto a 5 ft 10% carbowax 20 M 80-100 WAW-DMCS 5830 column. The column was heated at 70° for 5 min and was heated further with a rate of 10°/min up to 120°; this temperature was maintained until all volatile products had been swept from the column.

A sample of triazene (50 mmol) suspended in 0.1 M phosphate buffer (1 ml) pH 7.2 in 5 ml capacity screw capped reacti-vials was allowed to decompose for 24 hr at 37°C, after the evacuation of the head space above the liquid. The gaseous sample (1 ml) was injected by pressure-lock syringe onto GC for acetaldehyde and other volatile products, which were confirmed by the retention times by comparison to authentic samples and their masses by GC MS. Immediately after the removal of the gaseous contents, 0.5 ml of dichloromethane was injected in to the vial and shaken thoroughly and the dichloromethane solution (2 µl) was injected for GC and GC/MS analyses.

(b) Identification of Nonvolatile Products

After the identification of volatile products the reaction mixture was lyophilized and the residue was purified by chromatography on silica using benzene/5% acetone as eluant. The products were identified by NMR and exact mass measurements.

CHAPTER III.

ALKYLATION AND STRAND SCISSION OF DNA BY 2-HALOALKYLTRIAZENES

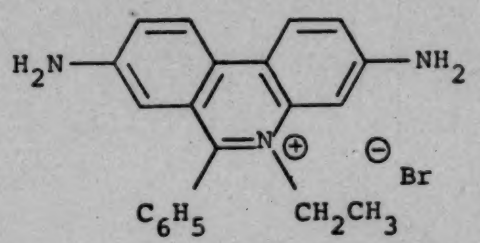
While considerable evidence indicates triazenes inhibit nucleic acid synthesis[59,60] little is known about their mechanism of action, in particular of their chemical interaction with cell target sites such as DNA. At first sight it appears that monoalkyltriazenes are similar to the clinically effective 2-haloethylnitrosoureas that decompose under physiological conditions giving rise to electrophiles[7,59-61] which attack biological macromolecules such as DNA. However, owing to the unique acid promoted decomposition of triazenes and base promoted decomposition of nitrosoureas[62] there lies a difference in the underlying chemistry of the two classes of compounds which became evident in their reactions with polynucleic acids as discussed in this chapter.

Studies Related to the Alkylation of DNA by Triazenes

DNA alkylation by triazenes was measured by their relative abilities to alkylate PM2 covalently-closed-circular-DNA (PM2-CCC-DNA) using the rapid and convenient ethidium fluorescence assay.

Ethidium bromide 39 is a trypanocidal dye that interacts with DNA. Le Pecq and Paoletti[63] as well as Morgan and Paetkau[40] have observed a marked increase in the

fluorescence of the dye in the presence of bihelical nucleic acids while no enhancement is observed in the presence of single stranded nucleic acids under conditions where regions of accidental self-complementarity are prevented. Le Pecq and Paoletti[63] concluded that the ethidium cation binds to duplex regions of nucleic acids by intercalation between base planes. Their results suggested that ethidium



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bromide binds once for every five nucleotides, a conclusion consistent with previous X-ray diffraction data.[64] They proposed that the fluorescence enhancement is due to the occlusion of the ethidium cation, by intercalation, into the hydrophobic region of the nucleic acids where it is protected against quenching by the aqueous solvent. This view was supported by experiments that showed that the fluorescence of ethidium bromide increased when it was measured in alcohols of decreasing hydrophilic character, ranging from ethylene glycol to octanol.[63]

Morgan and Paetkau observed,[40] that when an ethidium bromide concentration of 0.5 μg/ml was employed, a linear response of fluorescence with bihelical DNA concentration up to 0.02 A_{260} was obtained. The observation that fluorescence is directly proportional to the amount of

double stranded DNA in solution has permitted the development of convenient assays for measuring (among other chemical lesions) alkylation of DNA.

Alkylation is detected with PM2-CCC-DNA. Using the ethidium fluorescence assay, aliquots of a reaction mixture containing DNA are analyzed for base alkylation by dilution with a solution of ethidium bromide buffered to pH 11.8. The fluorescence of the DNA-ethidium solution provides an estimate of the total DNA concentration. The DNA in ethidium solution is heat denatured (96°C/3min) and cooled and equilibrated to 22°. Under these conditions unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints whereas alkylated PM2-CCC-DNA undergoes a facile depurination or depyrimidination in the reaction mixture or during the heat denaturation to produce AP sites which hydrolyze quickly in the hot alkaline solution. The resulting open circular DNA (OC-DNA) heat denatures to form one circular strand and one linear strand which under the pH 11.8 conditions do not bind ethidium bromide, and as a result the fluorescence falls to zero. The ratio of the decrease in fluorescence (after the heat denaturation and cooling cycle) to that of the control is a measure of the extent of alkylation. The basis of the assay is illustrated in Figure 1.

Alkylation measured with the ethidium bromide fluorescence assay was observed for all of the triazenes prepared in this study. Typical examples of alkylation are shown in

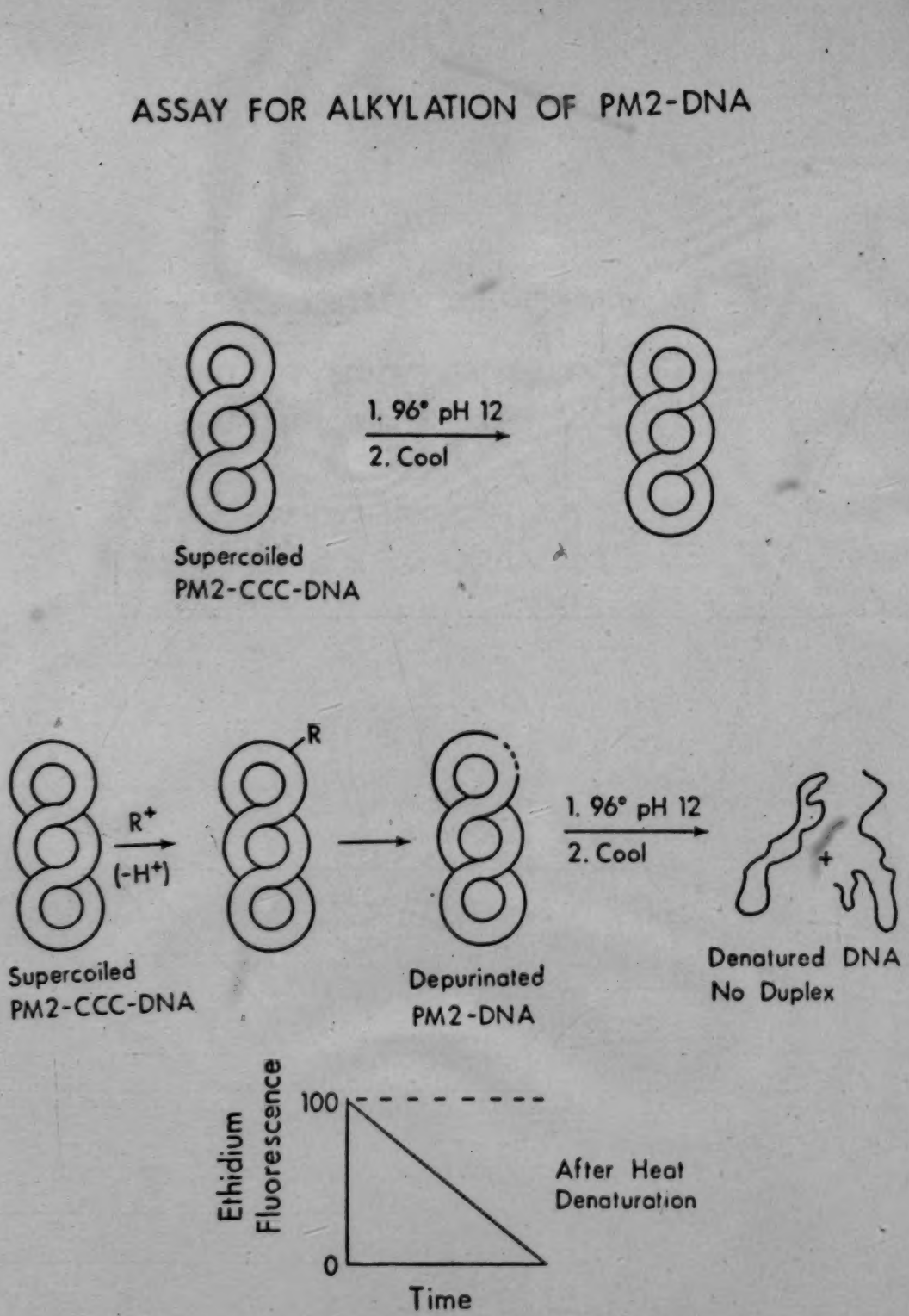


Figure 1. Ethidium bromide assay for DNA base alkylation of PM2-DNA.

Figure 2 and Table 5. The results are in accord with previous reports of *in vitro* alkylation of nucleic acids and nucleotides by phenylmonomethyl triazenes[65,66] and by *in vitro* treatment of calf thymus DNA with tritium labelled MlC.[67] With the exception of those two compounds run in aqueous dimethyl sulfoxide one may discern in Table 5 a trend favouring greater DNA alkylation for triazenes that decompose more rapidly. However, some exceptions indicate a complex dependence of structure on the two different types of reactivity. The parallel is most clearly discerned within a group of triazenes when stability of the aryltriazene is governed largely by the nature of the alkyl group in the side chain (e.g. 15, 16, 34 and 25). An apparent exception to this general trend is in the case of 1-(*p*-cyanophenyl)-3-(2-chloropropyl)triazene 34 with a decomposition half life of 1494 sec. Compound 34 alkylates DNA at a much slower rate than does 1-(*p*-cyanophenyl)-3-(2-fluoroethyl)triazene 15 which has a comparable $t_{\frac{1}{2}}$ of 1392 sec. It is possible that the potentially alkylating electrophiles generated from 34 are removed to a greater extent by, for example, proton loss and hydride transfer (Scheme 7). The relative stabilities of *ortho* nitro substituted triazenes (where pmr evidence indicates freezing of one tautomer by intramolecular hydrogen bonding, see previous chapter) and the *para* nitro substituted isomer is also reflected in their relative rates of DNA alkylation (Figure 2).

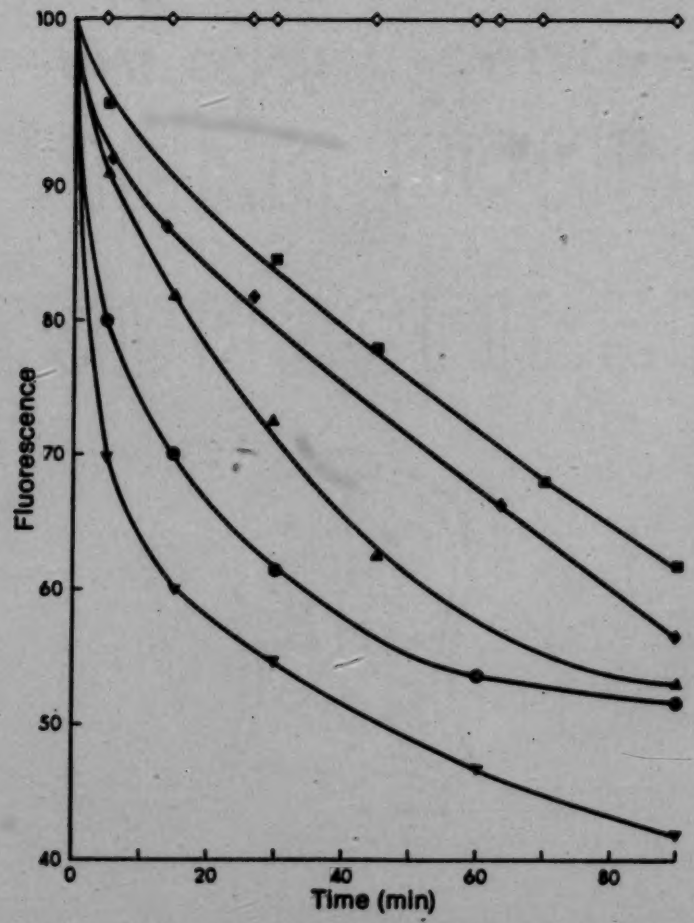


Figure 2. Thermally induced DNA strand scission resulting from alkylation by 3-(2-haloethyl)aryltriazenes. Reaction of 1 mM drug in 10% aqueous acetonitrile with 1.0 A_{260} unit of PM2-CCC-DNA pH 7.0, 37°. Filled symbols are fluorescence readings after heat denaturation at 96°/3 min followed by cooling to 22° (■ - ■) 1-(p-cyanophenyl)-3-(2-bromoethyltriazene 17; (◆ - ◆) 1-(p-nitrophenyl)-3-(2-fluoroethyl)triazene 12; (▲ - ▲) 1-(p-carboethoxyphenyl)-3-(2-fluoroethyl)triazene 20; (● - ●) 1-(o-nitrophenyl)-3-(2-fluoroethyl)triazene 22; (▼ - ▼) 5-[3-(2-fluoroethyl)-1-triazenyl)]imidazole-4-carboxamide 35. (◇ - ◇) control reaction.

TABLE 5

RELATIVE EXTENTS OF ALKYLATION OF DNA AND RATES OF AQUEOUS DECOMPOSITION OF 3-(2-HALOETHYL)ARYLTRIAZENES

$$p\text{-}R_1\text{-}C_6H_4\text{-}N{=}N\text{-}NH(CH_2)_n\overset{R_2}{\underset{|}{C}}H\text{-}R_3$$

Compound	n	R_1	R_2	R_3	% Loss of Fluorescence 37°, pH 7.2 in 15 min[a]	$t_{\frac{1}{2}}$ (sec)[a]
18	1	CH_3CO	H	F	23	2304
20	1	EtOCO	H	F	29	1863
20	1	EtOCO	H	F	19[b]	1522[b]
34	1	CN	CH_3	Cl	9	1494
15	1	CN	H	F	19	1392
25	3	CN	H	Cl	21	972
35		(Imidazole)		F	40[b]	704[b]
24	2	CN	H	Cl	24	396
27	2	CH_3CO	H	Cl	33	264
21	1	EtOCO	H	Cl	48	164
16	1	CN	H	Cl	30	104
19	1	CH_3CO	H	Cl	41	<1

[a] 1 mM drug in 10% aqueous CH_3CN unless otherwise stated. $t_{\frac{1}{2}}$ indicates time for 50% decomposition.

[b] In 10% aqueous dimethyl sulfoxide.

The 3-(2-haloethyl)aryltriazenes are very labile compounds and (in contrast to the corresponding nitrosoureas)[68] their rates of decomposition increase with decreasing pH of the medium as discussed in Chapter II. The concomitant increased rate of generation of electrophiles is reflected in a parallel increase in the rate of DNA alkylation in the pH range 10 to 6 (Table 6).

Triazenes Induced DNA Single Strand Scission (SSS)

The fluorescence assay described in this chapter can also be adapted to detect single strand scission of covalently closed circular DNA (CCC-DNA). The amount of ethidium bromide 39 taken up by supercoiled PM2-CCC-DNA is restricted due to topological constraints. Single strand scission of CCC-DNA results in the production of open circular DNA (OC-DNA) in which the topological constraints are removed. Depending on the superhelix density of the parent CCC-DNA the OC-DNA can take up to about 30% more ethidium than negatively supercoiled CCC-DNA with a corresponding increase in fluorescence observed.

As described earlier PM2-CCC-DNA returns to register and to the initial ethidium fluorescence intensity after the heating (96°C/3 min) and cooling (22°C) cycle whereas PM2-OC-DNA, upon heat denaturation forms one linear and one circular strand. Thus strand separation removes the duplex regions under pH 11.8 conditions which is reflected in the fall of fluorescence. This sequence of events is illustrated in Figure 3.

TABLE 6

EFFECTS OF pH OF THE MEDIUM ON THE EXTENT OF ALKYLATION OF DNA AND RATES OF AQUEOUS DECOMPOSITION OF 3-(2-FLUOROETHYL)-P-CYANOPHENYLTRIAZENE 15

Medium pH	% Loss of Fluorescence 37° in 15 min	$t_{\frac{1}{2}}$ 26°, (sec)
10	0	18000
8	10	10080
7	19	7549
6	29	3175

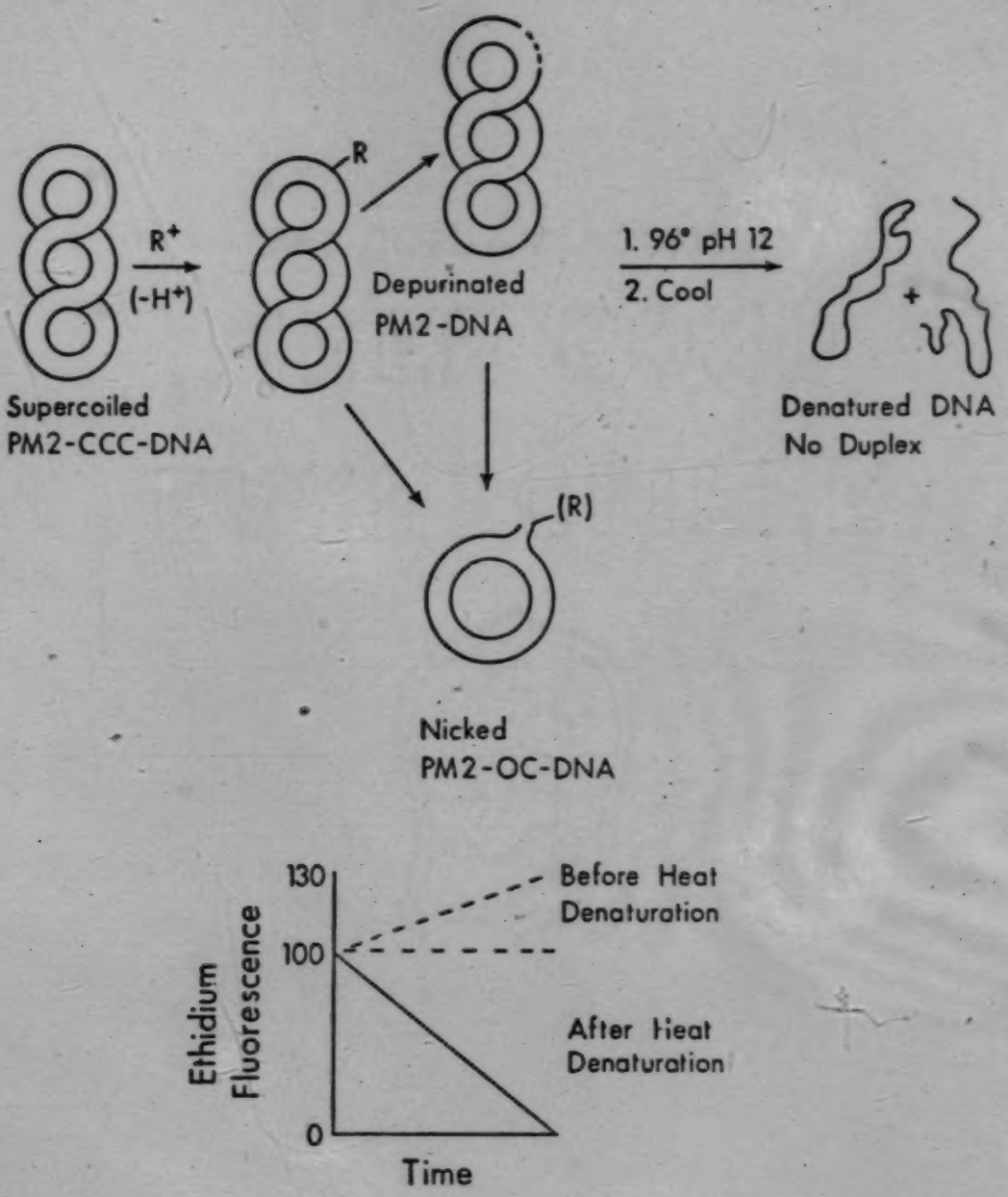


Figure 3. Ethidium bromide assay for the detection of single strand scission of supercoiled PM2-CCC-DNA.

The relative increase in fluorescence upon nicking the DNA can be further enhanced by initially treating the PM2-CCC-DNA with calf thymus topoisomerase.[117] Native PM2-CCC-DNA contains negative super coils.[118] The topoisomerase by acting as both an endonuclease and a ligase removes the super-coils to relax the DNA.[117] During this process the number of intercalation sites for ethidium (which itself unwinds the supercoiled PM2-CCC-DNA) is decreased. The relaxation process can be monitored by a 25-35% decrease in fluorescence. The conversion of relaxed PM2-CCC-DNA to PM2-OC-DNA now results in relative 80-100% increase in fluorescence which consequently increases the sensitivity of the assay. The use of the ethidium bromide fluorescence assay in conjunction with calf thymus topoisomerase is illustrated in Figure 4.

Single strand breaks are conventionally detected by gel electrophoresis or by alkaline sucrose gradient sedimentation. A particular advantage of employing the alternative ethidium assay however is that it permits a discrimination between two distinct types of DNA single strand scission as was observed initially with the 2-haloethyl-nitrosoureas. Type I SSS[69] is relatively rapid and is attributed to alkylation of the phosphate residues followed by assisted tri-ester hydrolysis. Type II SSS[69] is relatively slower and (owing to the selective effect of AP site specific endonuclease VI) is ascribed to base alkylation, depurination or depyrimidination, then base

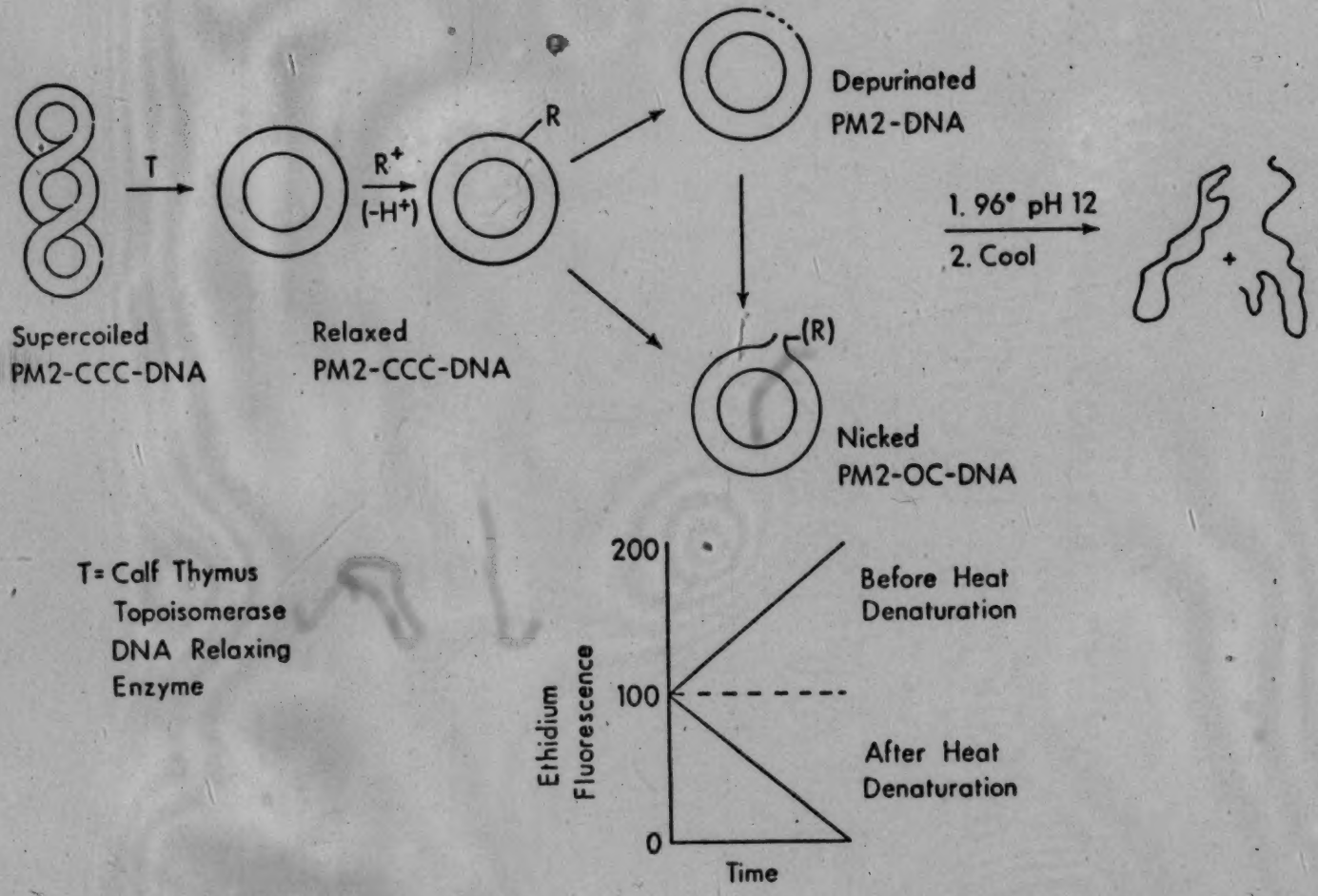


Figure 4. Ethidium bromide assay for the detection of single strand scission of PM2-CCC-DNA relaxed with calf thymus topoisomerase.

opening of the AP site.

A slow production of alkali labile sites which result in single strand breaks in DNA is observed after treatment of relaxed PM2-CCC-DNA with triazenes. The reaction mixture was incubated at pH 7, 37° and assayed at pH 11.8 buffer (which quenches further reaction of the triazene with the DNA) (Figure 5). As has been observed earlier with 2-haloethylnitrosoureas[69] controls verified that untreated DNA is stable under the high pH conditions for 120 min. Under certain conditions (e.g. in the presence of adventitious traces of metal ions) triazenes can undergo decomposition to give free radicals.[70] However in the present case the slow strand scission assayed at pH 11.8 was unaffected by prior treatment at pH 7.0 with superoxide dismutase, by catalase, or by free radical scavengers such as isopropyl alcohol or sodium benzoate or by EDTA. These observations rule out a free radical mechanism for the DNA strand scission such as operates for bleomycin or anthracyclines in the presence of reducing agents.[71,72]

Origin of Single Strand Breaks Produced by 2-Haloethylaryltriazenes

Having excluded a free radical process for the SSS of DNA and in the absence of evidence for Type I SSS it seemed probable that the 3-(2-haloethyl)aryltriazenes were giving rise to strand breaks primarily as a result of Type II SSS consisting of base alkylation, followed by depurination or

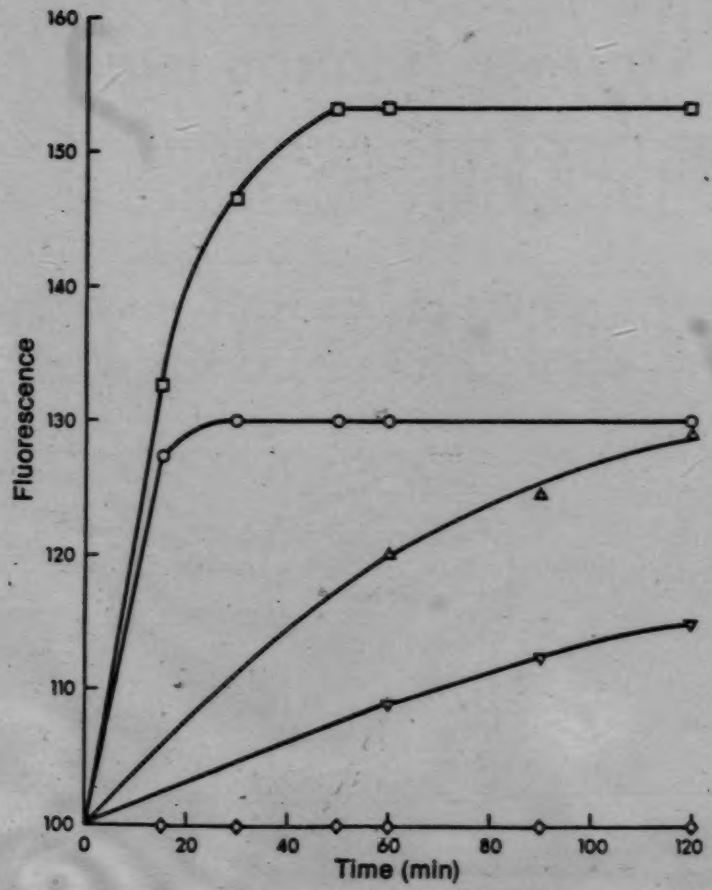


Figure 5. Confirmation of Type II SSS induced in DNA by 3-(2-haloethyl)aryltriazenes with site specific repair enzyme and with alkali incubation. Reaction of 1 mM drug with 1.0 A_{260} unit of topoisomerase relaxed PM2-CCC-DNA at pH 7.0, 37°. Open symbols are fluorescence readings before heat denaturation at 96°/3 min taken after 60 min incubation with the drug followed by treatment with either endonuclease VI or alkali assay at pH 11.8 (□-□) 1-(p-cyanophenyl)-3-(2-chloroethyl)triazene 16 and incubation with endonuclease VI; (Δ-Δ) triazene 16 and incubation at 37° at pH 11.8; (o-o) 1-(p-carboethoxyphenyl)-3-(2-fluoroethyl)triazene 20 and incubation with endonuclease VI: (▽-▽) triazene 20 and incubation at 37° at pH 11.8. (○-○) control reaction.

or depyrimidination and subsequent hydrolysis of the apurinic sites produced. A monomethyltriazene has been observed to alkylate the guanine residue of calf thymus DNA *in vitro* probably at the N-7 position.[66] In addition the facile loss of alkylated bases from the modified DNA polymer to generate apurinic sites is well documented.[73,74] The postulate of Type II SSS induced by triazenes was confirmed by using the apurinic site specific enzyme endonuclease VI[69] on the triazene treated DNA. A prompt and extensive production of SSS was observed (Figure 5) confirming apurinic sites produced by the 2-haloethyltriazenes.

Monoalkyltriazenes in general alkylate acidic sites on DNA more effectively than basic sites.[75] Also our observation that 3-(2-haloethyl)aryltriazenes readily esterify diethylphosphate (see experimental) indicates that the alkylation of the phosphate residues of nucleic acids by these triazenes could be a significant event both *in vivo* and *in vitro*. Previous studies on 2-haloethylnitrosoureas had shown that 2-chloroethylation of the phosphate backbone does not give rise to extensive Type I SSS, but that 2-hydroxyethylation leads to assisted hydrolysis giving very rapid SSS[69,76-78] (Figure 6). Since aqueous decomposition of 3-(2-haloethyl)aryltriazenes gives rise to substituted anilines and since apurinic nucleic acids have been reported to be cleaved by aromatic amines via a Schiff base intermediate[79,80] the possible contribution of this pathway had

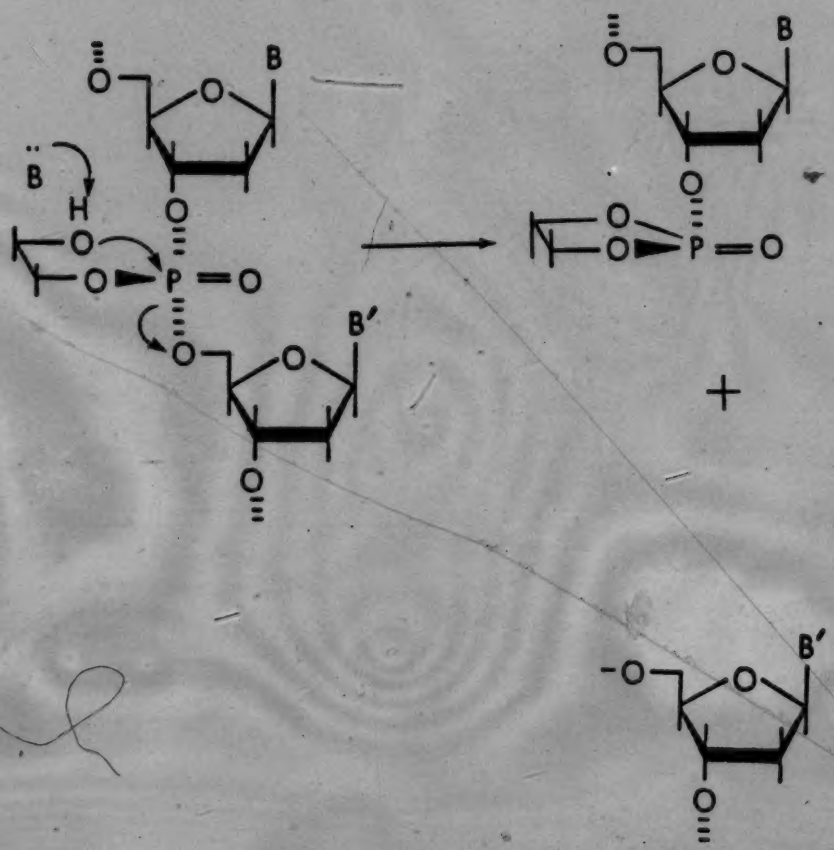
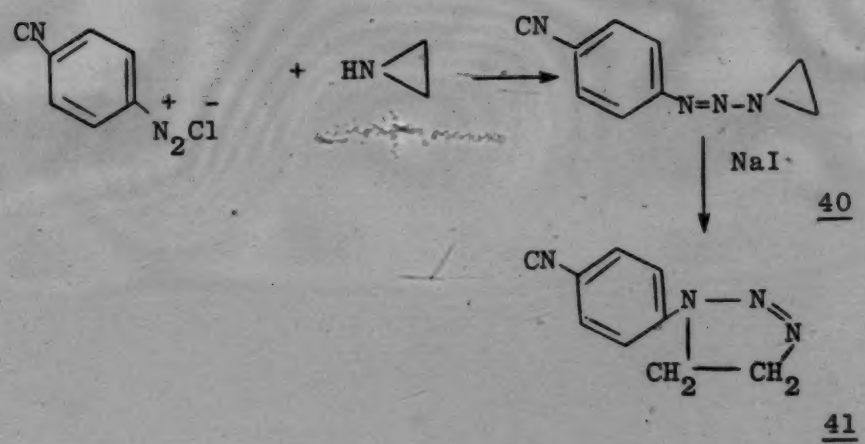


Figure 6. Hydrolysis of a DNA phosphotriester catalyzed by the 2-hydroxyethyl group.

to be considered. In the study of this effect in the case of 2-haloethylnitrosoureas this is only significant in the case of anilines bearing electron releasing groups, e.g. *p*-methoxyaniline, and even in such cases is unlikely to be competitive *in vivo* with the operation of specific AP endonucleases.[69] The anilines released from the present aryltriazenes are without exception those containing electron-withdrawing groups so that their effects on AP sites produced by the triazenes may be neglected. The intermediacy of 1,2,3-triazoline implicated in the aqueous decomposition of (2-haloethyl)aryltriazenes has been discussed in Chapter II. It was considered appropriate to synthesize a representative triazoline such as 41 and study its chemistry in aqueous solution. Compound 41 was synthesized from the corresponding phenylazoaziridine 40 (Scheme 10).

Scheme 10



The triazoline 41 was incubated at 37° and at pH 7 in the presence of sodium chloride for 24 h, the products were separated by silica gel chromatography and identified as *p*-N-(2-chloroethyl)aminobenzonitrile and *p*-N-(2-hydroxyethyl)aminobenzonitrile. Since these products were also obtained from the corresponding triazene 16, this together with other evidence (primarily from deuterium labelling studies described in Chapter II) supported the intermediacy of the triazoline in the decomposition of 16 (Scheme 6).

Having identified the triazoline 41 as a key intermediate in the aqueous decomposition of 16, it seemed possible that, depending on the leaving group ability of the halogens in the side chain (Br>Cl>F), the triazolines could be formed from 2-haloethyltriazenes to varying extents in aqueous solutions. Therefore the reaction of triazoline 41 with relaxed PM2-CCC-DNA was studied in order to delineate the contribution of this type of intermediate to the overall reactions of 2-haloethyltriazenes with DNA. The triazoline 41 was incubated with PM2-CCC-DNA at 37°, pH 7 and the progress of the reaction was monitored using the ethidium fluorescence assay for 2 hrs. During this period only a slow and progressive alkylation of DNA was observed without DNA single strand scission of either Type I or Type II (Figure 7).

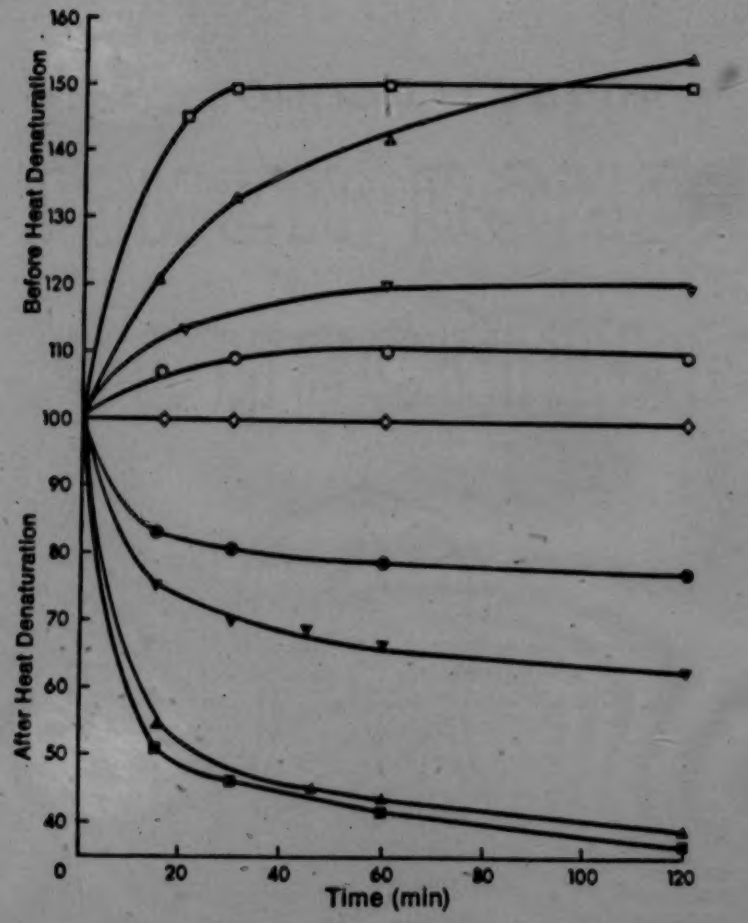
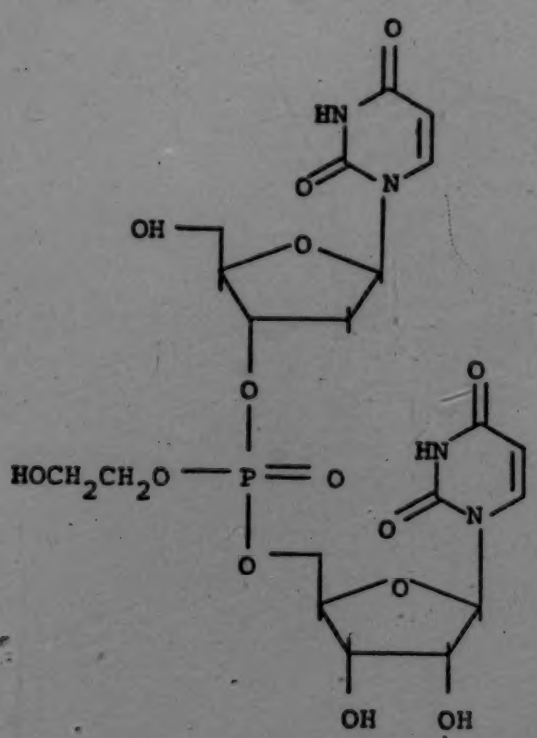


Figure 7. Type I and type II SSS induced in DNA by different 3-(2-haloethyl)aryltriazenes. Reaction of 1 mM drug in 10% aqueous acetonitrile with 1.0 A_{260} unit of PM2-CCC-DNA at pH 7.0, 37°. Open symbols are fluorescence readings before heat denaturation and indicate rapid Type I SSS. Closed symbols are fluorescence readings after heat denaturation at 96°/3 min followed by cooling to 22° and reveal Type II SSS confirmed by the specific effect of endonuclease VI. (□-□) 1-(p-cyanophenyl)-3-(2-hydroxyethyl)triazene 43; (Δ-Δ) 1-(p-carboethoxyphenyl)-3-(2-hydroxyethyl)-triazene 45; (o-o) 1-(p-cyanophenyl)-3-(2-methoxyethyl)triazene 44; (▽-▽) 1-(p-carboethoxyphenyl)-3-(2-methoxyethyl)triazene 46; (◇-◇) triazoline 41 or control reaction.

Hydroxyethylation of DNA and Concomitant Type-1 Strand Scission

It has been reported that hydroxyethyl phosphotriesters of DNA result in strand scission under neutral conditions.[77,78] However, there is some disagreement concerning DNA strand scission after hydroxyethylation.[81] In a model study conducted on the β-hydroxyethyl phosphotriester of the deoxyuridylyl-(3'-5')-uridine dinucleotide



42

42 it was observed that the nucleotide is stable at pH 7.5 and 40°C but will readily undergo base catalyzed hydrolysis in aqueous ammonia at 20°C to yield a mixture of nucleotide products.[82]

In order to investigate the alkylation of the phosphate backbone of DNA by monoalkyltriazenes in general the following two experiments were designed:

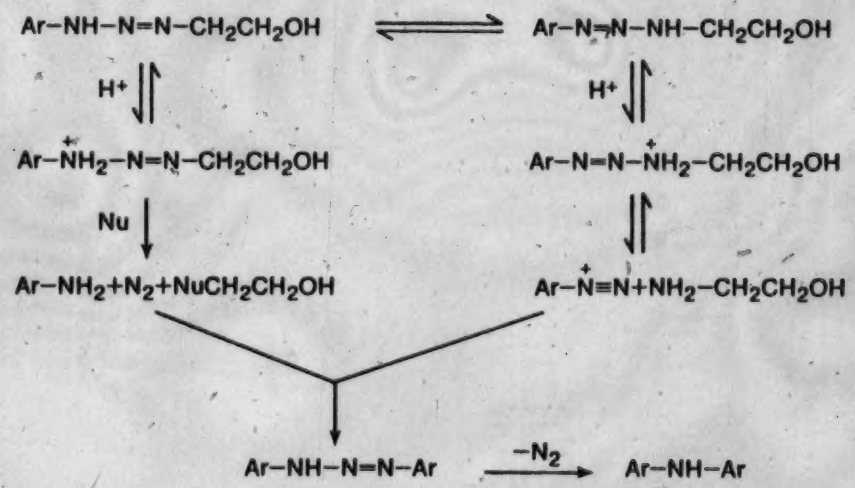
(1) 3-(2-hydroxyethyl)aryltriazenes 43 and 45 were prepared and their chemistry in aqueous solution as well as their reaction with DNA was studied.

When 1-(*p*-cyanophenyl)-3-(2-hydroxyethyl)triazene 43 was allowed to decompose at 37° in the presence of chloride ion, acetaldehyde and 2-chloroethanol (1:2) were identified by GC/MS. In the absence of chloride ion, ethylene glycol was the major product.

Similar aqueous decomposition at pH 7.2, 37° of 1-(*p*-carboethoxyphenyl)-3-(2-hydroxyethyl)triazene 45 in the presence of chloride ion afforded acetaldehyde and 2-chloroethanol in a ratio of (1:2). The involatile products included ethyl *p*-aminobenzoate and the diarylamine. The latter products, accounting for 30% of the involatiles, may arise as shown in Scheme 11.

It is evident from the products of aqueous decomposition that 2-hydroxyethylaryltriazenes decompose to generate potential electrophiles in a manner similar to what has been observed with 2-haloalkylaryltriazenes discussed earlier.

Scheme 11


Ar–NH–N=N–CH2CH2OH
Ar–N=N–NH–CH2CH2OH
H+
H+
Ar–NH2–N=N–CH2CH2OH
Ar–N=N–NH2–CH2CH2OH
Nu
Ar–NH2+N2+NuCH2CH2OH
Ar–N≡N+NH2–CH2CH2OH
Ar–NH–N=N–Ar
–N2
Ar–NH–Ar

Triazenes 43 and 45 caused rapid and extensive Type I SSS when incubated with PM2-DNA (Figure 7). This observation indicates an efficient hydroxyethylation of the phosphate residue of DNA resulting in strand scission. Under similar conditions and as a control the corresponding 2-methoxyethylaryltriazenes 44 and 46 gave no evidence of extensive Type I SSS but, like the triazoline only a slow and progressive alkylation. This can not be attributed to differences in rates of decomposition and thereby of electrophile generation since structurally related pairs of (2-hydroxyethyl) and (2-methoxyethyl)triazenes decompose at comparable rates as measured polarographically (Table 7).

Since no Type I strand scission was observed either with 2-chloroethyltriazene 16 or the corresponding triazoline 41 it was concluded that although 3-(2-haloethyl)-aryltriazenes decompose in part *via* an intermediate triazoline the latter while giving rise to a 2-hydroxyethylaniline by ring opening and loss of nitrogen does not generate an appreciate concentration of 3-(2-hydroxyethyl)aryltriazene.

(2) Assessment of Nucleic Acid Phosphate Group Alkylation by 3-(2-haloethyl)aryltriazenes Employing RNA.

The results discussed above implied but did not prove that treatment of DNA with 3-(2-haloethyl)aryltriazenes (or monoalkyltriazenes in general) leads to alkylation of the phosphate residues. Ethyl and methyl phosphotriesters

TABLE 7

POLAROGRAPHIC BEHAVIOR OF SELECTED ARYLTRIAZENES

$p\text{-}R_1\text{-}C_6H_4\text{-}N{=}N\text{-}NR_2\text{-}CH_2CH_2R_3$

Compound	R_1	R_2	R_3	$E_{\frac{1}{2}}$(V)[a] vs S.C.E.	$t_{\frac{1}{2}}$ (sec)[a]
43	CN	H	OH	-0.925	576
44	CN	H	OCH_3	-0.945	756
45	CO_2Et	H	OH	-0.926	1302
46	CO_2Et	H	OCH_3	-0.942	1650
52	CN	H	H	-0.930	397
53	CN	(CH_2OH)	(H)	-0.922	399

[a]Determined in 10% aqueous acetonitrile.

of DNA are stable under neutral conditions[83] and ethyl phosphotriesters hydrolyze only very slowly in 0.1 N NaOH.[84] RNA internucleotide linkages are much less stable and the glycosidic linkages much more stable than those of DNA. Phosphotriesters of ribonucleotides are unstable over the entire pH range presumably due to participation in the hydrolysis step by the 2'-hydroxyl group on the sugar moiety (Figure 8).[85] This property permits the observation of RNA degradation by alkylating agents to be used as a diagnostic test for phosphotriester formation.[36,76] Alkylation of the base residues of RNA produces a more stable system than in DNA and therefore, depurination or depyrimidination of alkylated bases followed by hydrolytic cleavage is less likely to contribute to RNA degradation.

The average molecular weight of poly A was determined before and after incubation with the 2-chloroethyltriazene 16 at 37° and pH 7.0 using sedimentation velocity changes measured on a Beckman analytical ultracentrifuge. The molecular weights were recorded as 162,000 and 87,000 respectively indicating an approximately 50% loss in molecular weight over a period of 1 h. The results suggest that extensive 2-chloroethylation of the phosphate residues of nucleic acids may contribute significantly in the mode of action of 3-(2-chloroethyl)aryltriazenes but that 2-hydroxyethylation of nucleic acids is not significant, in contrast to the case of 2-haloethyl nitrosoureas.[69] Such hydroxyethylation of nucleic acids, if it had occurred, would have resulted in extensive Type I SSS as

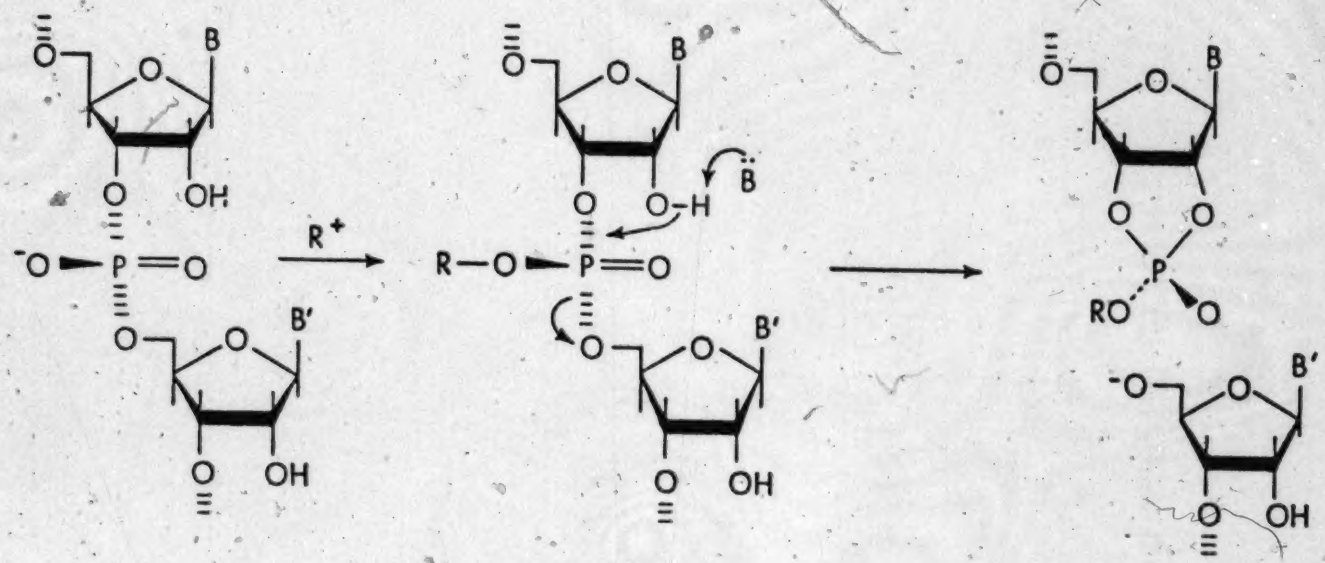


Figure 8. Hydrolysis of an RNA phosphotriester catalyzed by the 2'-hydroxyl group fo the ribose.

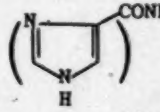
in the case of reaction by 3-(2-hydroxyethyl)aryltriazenes 43 and 45 described earlier and shown in Figure 7.

In conclusion, the evidence suggests that 3-(2-haloethyl)aryltriazenes generally undergo chemical transformations under physiological conditions to generate electrophiles including the 2-chloroethyl cation and, as a minor pathway, the 1-aryltriazoline. Because of the unique acid promoted decomposition of triazenes which may lead to preferential reaction in tumor tissue (which has slightly lower pH than normal tissue[43,44] this may result in preferential and extensive alkylation of the DNA phosphate residues although the bases are also alkylated) resulting in single strand scission largely of Type II. Several of these new types of aryl triazenes, especially the 2-fluoroethyl derivatives 18, 20, and 35 exhibit promising antileukemic properties in animal test systems (Table 8). A brief comment on the remarkable activity of 2-fluoroethyltriazenes is warranted. It is possible that 2-fluoroethyltriazenes, being the most stable within a group of 2-haloethyltriazenes, reach the macromolecular cell targets intact and with a minimum loss of potential electrophiles, for example, via triazoline formation, alkylate the macromolecules most efficiently. However, the lower activity of other stable monoalkyl triazenes may be related to the factors such as solubility and lypophilicity etc.

TABLE 8

ACTIVITY OF 2-HALOETHYLARYLTRIAZENES AGAINST P388 LEUKEMIA[a]

$p\text{-}R_1\text{-}C_6H_4\text{-}N{=}N\text{-}NH(CH_2)_n\overset{R_2}{\underset{|}{C}}H\text{-}R_3$

Compound	n	R_1	R_2	R_3	Dose (mg/kg) /day	Weight Diff.[b] (T-C)	CNTL Body change[c]	Survivors on day 5	mean survival time/days		
									Test	Contl.	% T/C
13	1	NO_2	H	Cl	25	-1.7	2.1	5/5	13.5	10.5	128
20	1	COOEt	H	F	25	-5.3	1.0	6/6	30.0	11.7	256
18	1	CH_3CO	H	F	12.5	-2.5	2.1	5/5	28.5	10.7	271
19	1	CH_3CO	H	Cl	100	-0.9	2.1	5/5	11.0	10.5	104
34	1	CN	CH_3	Cl	20	-2.5	1.5	5/6	12.3	10.3	119
27	2	CH_3CO	H	Cl	50	-0.9	2.1	5/5	13.3	10.5	125
52	1	CN	H	H	50	-2.4	2.1	3/3	13.0	10.5	123
35			H	F	3.13	-2.8	1.7	6/6	19.0	10.6	179
53	1	CH_3CO	H	H	100	-3.7	2.1	5/5	14.5	10.5	138
43	1	CN	H	OH	20	-3.6	2.3	6/6	13.8	10.7	128
44	1	CN	H	OCH_3	25	-1.5	2.3	6/6	11.3	10.7	105
45	1	COOEt	H	OH	20	-5.1	2.3	6/6	12.7	10.7	118
46	1	COOEt	H	OCH_3	100	-5.5	2.3	6/6	14.8	10.7	138
15	1	CN	H	F	25	-5.1	1.0	4/6	16.0	11.7	136
16	1	CN	H	Cl	50	-2.7	2.1	5/5	15.0	10.5	142
24	2	CN	H	Cl	100	-2.3	1.3	5/6	13.7	11.3	121
25	3	CN	H	Cl	50	-6.5	1.0	6/6	12.3	11.7	105
26	4	CN	H	Cl	25	-0.8	1.0	6/6	12.3	11.7	105

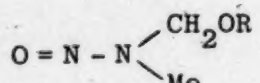
[a] Assays for activity against lymphoid leukemia P388 performed according to specifications established by the Cancer Chemotherapy National Service Centre.[111] Suspensions of the compounds in Saline and Tween 80 were administered intra-peritoneally within 5 min of preparation of suspensions. P388 cells (10^5) were implanted intra-peritoneally in mice on day 0.

[b] Average weight change of treated mice minus average weight change of control mice for a given dose.

[c] Average weight change of control animals in grams (weigh day 2 minus weigh day 1).

α-Hydroxylated Derivatives of Antitumor Dialkyltriazenes

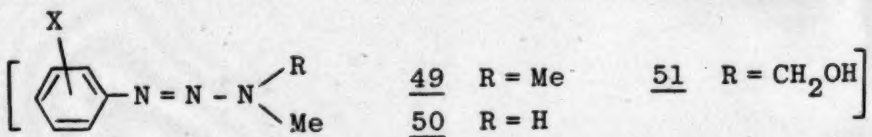
The suggestion[86] that metabolic α-hydroxylation could play a critical role in the carcinogenic activity of dimethyl nitrosamine (or in the expression of anticancer properties of 2-haloethylnitrosoureas) has been supported by the recent development of synthetic routes to α-acetoxydimethylnitrosamine 47.[87] Unlike dimethylnitrosamine the acetoxy derivative is markedly mutagenic without



47 R = Ac
48 R = H

prior metabolic activation.[88-90] Chemical studies with a range of α-acetoxymethylnitrosamines corroborate the theory that such compounds are non-enzymatically deacetylated to unstable hydroxymethyl derivatives which decompose to afford alkylating species.[87,91,92]

A similar oxidative activation process has been proposed to explain the fact that 1-aryl-3,3-dimethyltriazenes 49 although inactive *in vitro* develop antitumor properties



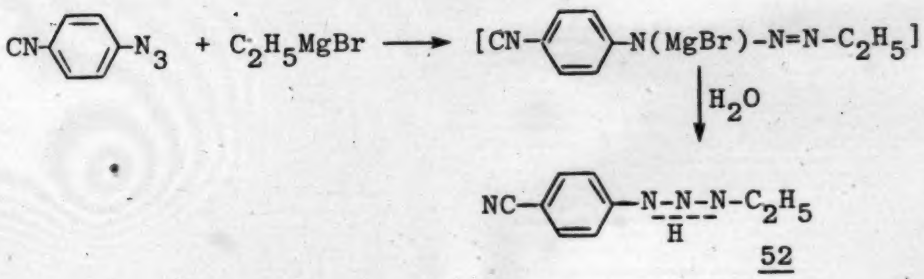
49 R = Me
50 R = H
51 R = CH_2OH

in vivo or after incubation with liver fractions.[4,93] In these cases alkylating monomethyltriazenes 50 are implicated as the cytotoxic species. Although the related α-hydroxymethyl intermediates 48 and 51 have been regarded as only

transient species until lately, K. Vaughan *et al.*[94] have developed a simple route to synthesize the latter type of compounds. Although ethylating triazenes have been found to be less active than methylating triazenes[94] a recent example of a monoethyl triazene 53' has shown a moderate antileukemic activity (Table 8). In order to gain insight into possible metabolic pathways the 3-ethyl-p-cyanophenyl-triazene 52 and 3-(hydroxymethyl-3-ethyl)p-cyanophenyl-triazene 53 were prepared.

It is noteworthy that triazene 52 could be prepared by controlled condensation of ethyl magnesium bromide with p-cyanophenylazide (Scheme 12) without significant interference by attack on the nitrile group, in contrast to an earlier report of the inapplicability of this procedure.[75]

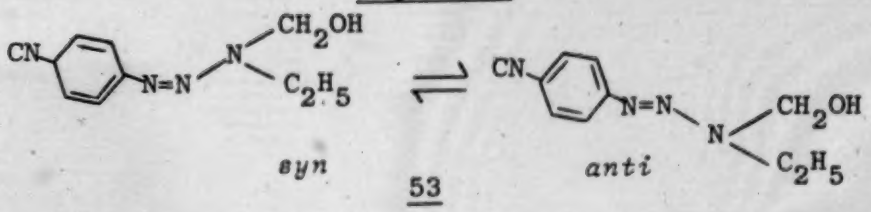
Scheme 12



52

It was also remarkable that the pmr spectrum of 53 shows two doublets at 5.05 δ and at -45° but only one doublet at 5.15 δ at 30° indicating rapid interconversion of the *syn* and *anti* triazene configurations at room temperature (Figure 9).

Figure 9



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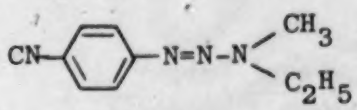
Compound 53 is a plausible primary oxidative metabolite of (3-ethyl-3-methyl)p-cyanophenyltriazene 54 and 54 in turn could give rise to 52. While 54 would be in-



54

active towards DNA in the absence of enzymatic activation, compounds 52 and 53 generate electrophiles and at comparable rates in accord with general Scheme 12. The half lives for decomposition in 10% aqueous CH_3CN of 52 and 53 are identical at 397 sec (Table 9). The volatile products of decomposition of 53 contained formaldehyde and ethanol (82% of volatile). The involatile products of 53 contained p-(N-ethylamino)benzonitrile and p-aminobenzonitrile. Similar aqueous decomposition of triazene 52 gave ethanol (100% of volatile products) and the two involatile products of 53.

When triazenes 52 and 53 were incubated with PM2-CCC-DNA at 37°, pH 7, and the progress of alkylation was monitored employing ethidium fluorescence assay, a progressive alkylation of DNA was observed by both triazenes at a comparable rate as shown in Figure 10.

TABLE 9

POLAROGRAPHIC BEHAVIOR OF MONOETHYLTRIAZENE 52

AND α-HYDROXYMETHYLETHYLTRIAZENE 53

	Reduction Potential	$t_{\frac{1}{2}}$ (sec)
# 52	-0.922	397
# 53	-0.940	397

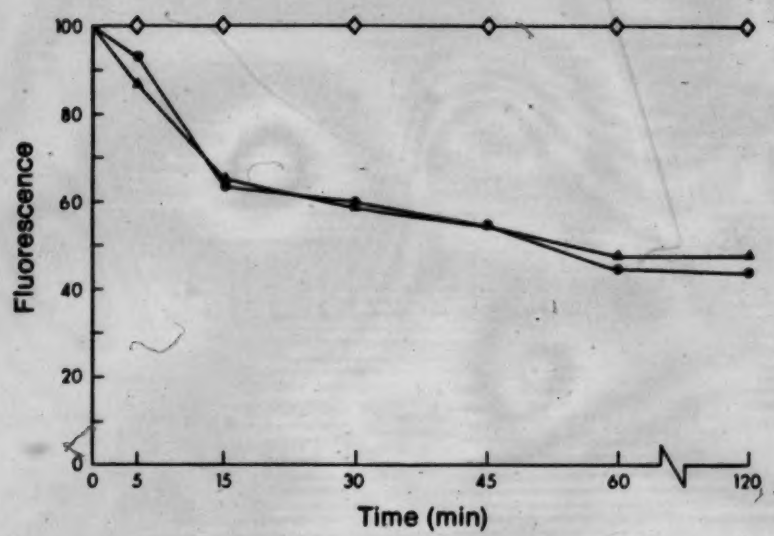
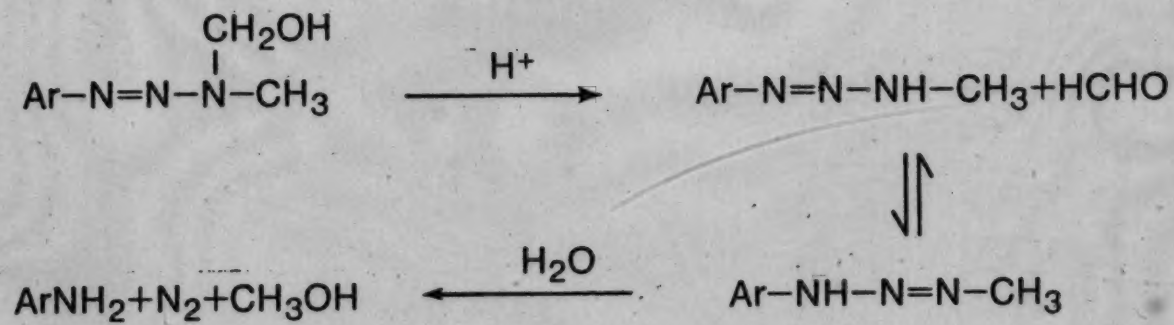


Figure 10. Thermally induced DNA strand scission resulting from alkylation. Reaction of 1 mM drug in 10% aqueous acetonitrile with 1.0 A_{260} unit of PM2-CCC-DNA pH 7.0, 37°. Symbols are fluorescence readings after heat denaturation at 96°/3 min followed by cooling to 22°. (▲-▲), 1-(p-cyanophenyl)-3-ethyltriazene; (●-●), 1-(p-cyanophenyl)-3-ethyl-3-(hydroxymethyl)triazene, (◇-◇), control reaction.

Scheme 12


CH2OH
Ar–N=N–N–CH3
H+
Ar–N=N–NH–CH3+HCHO
Ar–NH–N=N–CH3
H2O
ArNH2+N2+CH3OH

EXPERIMENTAL

The following triazenes were prepared by following the general procedure described for the 2-haloalkyltriazenes in Chapter II.

1-(p-Cyanophenylazo)aziridine 40

The neutralized diazonium salt solution was filtered and the filtrate cooled to -5°. To the stirred solution was added aziridine, in slight excess, dropwise. After stirring the mixture for 5 minutes the solid product was collected by filtration, washed with cold water and purified by rapid recrystallization from CH_2Cl_2 by cooling in a solid CO_2-acetone bath and adding petroleum ether dropwise. This yielded the triazene as an off white solid m.p. 45° (60% yield). P.m.r. [$CDCl_3$, -20°], 2.3 (s, 4H, aziridine ring protons), 7.2 - 7.7 (m, 4H, aromatic).

1-(p-Cyanophenyl)-Δ^2-1,2,3-triazoline 41

The azoaziridine 40 was isomerized following the procedure of Heine and Tomalia.[114] The resulting solid product was purified by recrystallization from methylene chloride and petroleum ether at low temperature as an off white solid m.p. 124° (95% yield). Anal. calcd. for $C_9H_8N_4$, 172.0749, M^+, 172.0757 (0.7%); Calcd. for $C_9H_8N_2$, 144.0688, M^+-N_2, 144.0685 (78%) (mass spectrum). P.m.r. [$CDCl_3$] 3.55 and 4.5 (2t, 4H, $-CH_2CH_2-$), 7.25 - 7.7 (m, 4H, aromatic). Ir ν_{max} ($CHCl_3$) 3223, 1610 cm^{-1}.

1-(p-Cyanophenyl)-3-(2-hydroxyethyl)triazene 43

This compound was obtained as a yellow solid which was purified by recrystallization from methylene chloride at -10° m.p. (dec.) 100° (90% yield). Anal. calcd. for $C_9H_{10}N_4O$, 190.0855, M^+, 190.0853 (7.5%); calcd. for $C_9H_{10}N_2O$, 162.0793, M^+-N_2, 162.0793, (1.6%) (mass spectrum). P.m.r. [$CDCl_3$] 2.15 (s, 1H, exch. OH), 3.9 (br, m, 4H, NCH_2CH_2O), 7.3-7.8 (m, 4H, aromatic), 9.5 (br, s, exch. NH). Ir ν_{max} 3466, 3288, 3261, 1609 cm^{-1}.

1-(p-Cyanophenyl)-3-(2-methoxyethyl)triazene 44

The product was obtained as a solid which was purified by recrystallization from methylene chloride at -30° by adding petroleum ether dropwise m.p. 81° (92% yield). Anal. calcd. for $C_{10}H_{12}N_4O$, 204.1012, M^+, 204.1012 (14%). Calcd. for $C_{10}H_{12}N_2O$, 176.0949, M^+-N_2, 176.0951 (1.5%) (mass spectrum). P.m.r. [$CDCl_3$-40°] 3.4 (s, 3H, OCH_3), 3.7 (t, 2H, CH_2O), 3.9 (t, 2H, NCH_2), 7.1 - 7.7 (m, 4H, aromatic), 8.6 and 9.6 (br, s, 2H, NH). Ir ν_{max} ($CHCl_3$), 3182, 3160, 1609 cm^{-1}.

The following triazenes were obtained as viscous oils which were extracted in CH_2Cl_2, dried ($MgSO_4$) and precipitated from the filtered solution by cooling in dry ice-acetone and adding petroleum ether dropwise.

1-(p-Carboethoxyphenyl)-3-(2-hydroxyethyl)triazene 45

A yellow solid m.p. 88-90° (60% yield). Anal. calcd. for $C_{11}H_{15}N_3O_3$, 237.1113, M^+, 237.1106 (1%); Calcd. for $C_{11}H_{15}NO_3$, 209.1052, M^+-N_2, 209.1047 (0.5%) (mass spectrum). P.m.r. [CD_2Cl_2] 1.35 (t, 3H, CH_3), 2.1 (br, s, 1H, exch. OH), 3.85 (br, s, 4H, $-NCH_2CH_2O^-$), 4.3 (t, 2H, OCH_2), 7.2 - 8.1 (m, 4H, aromatic), 9.2 (br, s, exch. NH). Ir ν_{max} ($CHCl_3$), 3504, 3229, 3188, 3162, 1715, 1688 cm^{-1}.

1-(p-Carboethoxyphenyl)-3-(2-methoxyethyl)triazene 46

A yellow solid m.p. 61-63° (92% yield). Anal. calcd. for $C_{12}H_{17}N_3O_3$, 251.1270, M^+, 251.1268 (20%); Calcd. for $C_{12}H_{17}NO_3$, 223.1208, M^+-N_2, 223.1206 (5%) (mass spectrum). P.m.r. [$CDCl_3$, -20°] 1.3 (t, 3H, CH_3), 3.4 (s, 3H, OCH_3), 3.75 (t, 2H, CH_2O), 3.95 (t, 2H, NCH_2), 4.35 (q, 2H, OCH_2), 7.2 - 8.2 (m, 4H, aromatic), 8.7, 9.9 (br, s, 1H, each, exch. NH). Ir ν_{max} ($CHCl_3$) 3225, 3190, 3171, 1702, 1687 cm^{-1}.

1-(p-cyanophenyl)-3-ethyltriazene 52

This triazene was prepared following an adapted procedure of Dimroth.[115] A solution of 1.44 g (10 mmol) of p-cyanophenylazide in 2 ml of anhydrous ether was added slowly to a solution of 11 mmol of ethylmagnesium bromide in 10 ml of anhydrous ether. After stirring the reaction mixture for 10 min a saturated solution of ammonium chloride

was added dropwise under cooling until the solid dissolved. The ether layer was removed, washed with water and dried ($CaCl_2$). After removal of the solvent the solid residue was purified by recrystallization from methylene chloride: petroleum ether at low temperature affording the triazene 52 m.p. 95-97° (76% yield). Anal. calcd. for $C_9H_{10}N_4$, 174.0905, M^+, 174.0907 (34%); calcd. for $C_9H_{10}N_2$, 146.0843, M^+-N_2, 146.0843 (3%) (mass spectrum). P.m.r. [CD_2Cl_2, -45°] 1.3 (m, 3H, CH_3), 3.7 (m, 2H, CH_2), 7.45 - 7.74 (m, 4H, aromatic), 8.35 and 9.65 (br, 2s, exch. NH). Ir ν_{max} ($CHCl_3$) 3184, 3160, 2219, 1608 cm^{-1}.

1-(p-Cyanophenyl)-3-ethyl-3-(hydroxymethyl)triazene 53

This triazene was prepared by adapting a procedure due to Vaughan and co-workers.[94] p-Aminobenzonitrile (1.18 g, 10 mmol) was diazotized at 0-5° in a mixture of 2.5 ml of concentrated hydrochloric acid and 10 ml of water using 800 mg of sodium nitrite dissolved in the minimum volume of water. The excess acid was neutralized with 1.6 g of $CaCO_3$ and the aqueous solution of the diazonium salt was added to a cold, premixed solution of 0.5 ml of ethylamine and (5 ml) of 40% aqueous formaldehyde. After stirring for 15 min the resulting precipitate was collected, washed with cold water, dried under vacuum and recrystallized from CH_2Cl_2 petroleum ether to afford triazene 53 as a white crystalline solid m.p. 65-66° (42% yield). Anal. calcd. for $C_{10}H_{12}N_4O$, N, 27.45%, Found, 27.15%. Calcd. mol. wt.,

204.1047, M^+, 204.1007 (0.4%), 174.0906, M^+-CH_2O, 146.0842, $M^+-CH_2O-N_2$, (mass spectrum). P.m.r. [CD_2Cl_2, -45°] 1.1 - 1.45 (m, 3H, CH_3), 3.65 - 4.0 (m, 2H, CH_2), 4.4 - 4.7 (m, 1H, OH), 5.0 - 5.3 (2d, NCH_2O), 7.3 - 7.7 (m, 4H, aromatic), [CD_2Cl_2, 30°], 1.3 (t, 3H, CH_3), 2.95 (br, s, 1H, exch. OH), 4.85 (q, 2H, CH_2), 5.15 (d, 2H, NCH_2O), 7.3 - 7.7 (m, 4H, aromatic). Ir ν_{max} ($CHCl_3$) 3432, 2228 cm^{-1}.

1-(p-Acetylphenyl)-3-ethyltriazene 53'

Yellow solid m.p. 82-83° (82% yield). Anal. calcd. for $C_{10}H_{13}N_3O$, 191.1058, M^+, 191.1056 (43%). Calcd. for $C_{10}H_{13}NO$, 163.0997, M^+-N_2, 163.0996 (7.5%) (mass spectrum). P.m.r. [$CDCl_3$, -45°] 1,3 (t, 3H, CH_3), 2.6 (s, 3H, OCH_3), 4.6 (q, 2H, NCH_2), 7.1 - 8.1 (m, 4H, aromatic), 8.2 and 10.2 (br, 2s, exch. NH). Ir ν_{max} ($CHCl_3$) 3216, 1667 cm^{-1}.

Preparation of diethyl 2-haloethylphosphates via esterification of diethyl phosphate with 2-haloethyltriazenes.

Diethyl, 2-fluoroethylphosphate

A typical reaction required the addition of 450 mg (3 mmol) of diethyl phosphate in 10 ml of ether to a suspension of 600 mg (3 mmol) of 5-[3-(2-fluoroethyl)-1-triazenyl]imidazole-4-carboxamide in 50 ml of anhydrous ether. The reaction mixture was protected from light and the stirring was continued for 12 h. After filtration and removal of the solvent *in vacuo*, the residue was chromatographed on

florisil using chloroform as eluant and affording 60 mg (10% yield) of triester. Anal. calcd. for $C_6H_{15}FO_4P$, 201.0692, M^+, 201.0704 (mass spectrum). P.m.r. τ[$CDCl_3$] 1.36 (t, 6H, $2CH_3$), 4.07 - 4.38 (m, 6H, OCH_2), 4.58 (2m, 2H, CH_2F). Ir ν_{max} ($CHCl_3$) 1470, 1440, 1380, 1360 and 1250 cm^{-1}.

2-Chloroethyl-diethyl phosphate

A solution of 1.5 g (10 mmol) of diethyl phosphate in 10 ml of ether was added to a stirred suspension of 2.5 g (12 mmol) of 1-(p-cyanophenyl)-3-(2-chloroethyl)triazene in 20 ml of anhydrous ether at room temperature. After 30 min the product was isolated and purified by chromatography on florisil using benzene/5% acetone as eluant yielding the triester 12 210 mg (8% yield). This material was identical to an authentic sample prepared by a literature procedure.[116]

Depurinated PM2-CCC-DNA

To 400 µl of PM2-CCC-DNA 8.0 A_{260} was added 25 µl 1 M sodium acetate buffer pH 3.05. The mixture was incubated at 37°C. 2 µl aliquots were withdrawn and added to the standard assay solution (which was 20 mM phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 µg/ml of ethidium) the fluorescence was measured and compared to that obtained after heating at 96°C/3 min and followed by rapid cooling.

Under these conditions unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological

constraints. Depurinated PM2-CCC-DNA shows a decrease in fluorescence due to alkaline strand scission of the apurinic site in the assay medium. The ratio of the decrease in fluorescence to that of the control is a measure of the extent of depurination. As long as the initial fluorescence reading remains constant, DNA degradation other than depurination is negligible. A previous study has shown[69] that a 90-120 minute incubation is necessary to introduce at least one apurinic site per molecule. After incubation, 50 µl of 1 M pH 7.2 phosphate buffer is added to quench the reaction. The solution of apurinic PM2-CCC-DNA may be stored for several days at 4°C.

Endonuclease Specific for AP Sites of Escherichia Coli (Endonuclease VI)

Endonuclease VI was purified according to Verly and Rassart[110] from *E. coli* BATCC 11303; after the phosphocellulose chromatography the enzyme was stored in 0.15 M NaCl, 0.04 M sodium phosphate pH 6.5 with an equal volume of glycerol and kept at -20°. For the experiments, this preparation was diluted with a suitable buffer.

Assay for Endonuclease VI Activity

The basis of the assay is that the enzyme cleaves AP PM2-CCC-DNA and thereby converts it to linear DNA which results in a change in ethidium fluorescence both before and after heat denaturation when measured at pH 8.0.

The reaction solution consisted of AP PM2-CCC-DNA 1.0 A_{260} unit in phtassium phosphate buffer pH 8.0. A 10 λ aliquot of the enzyme was added and the reaction solution incubated at 37°C for 15 min and the fluorescence of the resulting PM2-OC-DNA read using the standard pH 8 ethidium assay. Conversion of PM2-CCC-DNA employed in this study to PM2-OC-DNA by the endonuclease VI resulted in a characteristic 30% increase in fluorescence as a result of the release of topological constraints. After heat denaturation at 96°/3 min, when the PM2-OC-DNA is converted into single strands, then rapid cooling to 22° the fluorescence was read again. An active endonuclease VI fraction is revealed by loss of fluorescence after heat denaturation. The control for the assay consisted of a similar reaction substituting native PM2-CCC-DNA.

METHODS

All fluorometric measurements were performed on a G.K. Turner and Associates Model 430 spectrofluorometer equipped with a cooling fan to minimize fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One-centimeter square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 100 x scale of medium sensitivity was generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22°C.

Fluorescence Determination of PM2-CCC-DNA by Triazenes

A 20 µℓ aliquot was taken at intervals from the reaction mixture [50 mM potassium phosphate, pH 7.2, 1.2 A_{260} units of PM2-CCC-DNA (90% CCC), 1 mM triazene in a total volume of 200 µℓ at 37°] and added to the standard assay mixture (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 µg/ml of ethidium). The fluorescence after heating at 96°/3 min followed by rapid cooling was compared with the initial value.

Under these conditions unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints. Alkylated PM2-CCC-DNA shows a decrease in fluorescence because of thermally induced depurination followed by alkaline strand scission of the apurinic site in the assay medium. The ratio of the decrease in fluorescence (after the heat denaturation and cooling cycle) to that of the control is a measure of the extent of alkylation. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence.

Ethidium Fluorescence Assay for Type I SSS of DNA

A 300 µℓ sample containing PM2-CCC-DNA 1.0 A_{260}, 50 mM sodium cacodylate buffer pH 7.0 and 400 mM NaCl was incubated at 37° with the topoisomerase 0.01 A_{260}. The fluorescence was monitored by transferring 20 µℓ aliquots into 2 ml of the pH 11.8 assay solution. When a 25-30% decrease

in fluorescence had been observed (typically requiring a 30 min incubation), a 2 mM concentration of the desired drug was introduced and the fluorescence again monitored using 2 µℓ aliquots in 2 ml of the pH 11.8 assay solution. Readings were taken within 30 sec after addition of the aliquot so that AP site hydrolysis does not contribute to the observation of Type I SSS.

Ethidium Fluorescence Assay for Type II SSS of DNA

After the fluorescence reading had been taken to determine Type I SSS the pH 11.8 assay solution containing the 20 µℓ aliquot of reaction mixture was incubated at 37°. At designated times, the solution was reequilibrated to 22° for the fluorescence reading. A gradual increase in fluorescence indicates a gradual formation of nicked DNA due to basic hydrolysis of the labile AP sites.

Detection of AP Sites

A 300 µℓ solution containing 2 mM drug, 50 mM sodium cacodylate pH 7.0 and relaxed PM2-CCC-DNA 1.0 A_{260} was allowed to react for 120 min while monitoring for Type 1 SSS. 20 µℓ of the AP endonuclease solution was then added (the amount was determined by previous experiments with low pH depurinated PM2-CCC-DNA). The fluorescence was then monitored using the standard pH 8 ethidium assay. The percent of fluorescence increase with respect to the fluorescence at time 0 min was corrected for dilution by

the enzyme solution.

Detection of Phosphate Alkylation by RNA Degradation

A 150 µℓ solution containing 4 mg/ml poly A (Sigma Mw 162,000), 150 mM sodium cacodylate buffer pH 7.0 and 150 mM of 1-(p-cyanophenyl)-3-(2-chloroethyl)triazene 16 was incubated for 1 h. The reaction was quenched in ice and dialyzed against 50 mM potassium phosphate pH 7.2, 100 mM NaCl, 1 mM EDTA in triply distilled water at 4° for 24 h. The dialysate was then diluted with the dialysis solution to 1.0 A_{260} and the sedimentation velocity determined on a Beckman Analytical Ultracentrifuge.

General Method for the Aqueous Decomposition of Aryltriazenes

All decompositions and analyses of the decomposition products were performed following the procedure given in Chapter II unless otherwise stated.

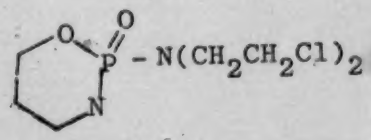
Identification of Ethylene Glycol in the Aqueous Decomposition of 42

GC Analysis was performed on a Hewlett-Packard Model 5840 A Gas chromatograph equipped with flame ionization detector. An aqueous solution of the decomposition products was injected onto a 6' column of porapak Q (80-100 mesh) maintained isothermally at 200°C with helium gas flow rate of 20 ml/min. The observed retention time for ethylene glycol was 6.5 min.

CHAPTER IV.

TRIAZENES AS TRANSPORT FORM OF SULFUR MUSTARD

Although bifunctionality is not a prerequisite for antineoplastic activity, the most active agents are bifunctional and have generally been observed[26] to be more lethal than their monofunctional counterparts. The greater efficacy of bifunctional alkylating agents with respect to cytotoxicity has been attributed to their ability to cross-link complementary strands of DNA. Nitrogen and sulfur mustards are two such bifunctional alkylating agents. Although the concept of using nitrogen mustards in various transport forms as anticancer agents has been investigated[95-98] with a certain degree of success[99] in the field of cancer chemotherapy, a parallel approach using sulfur mustards has not received such attention. Selective cytotoxicity of N-phosporylated nitrogen mustard such as cyclophosphamide (cytoxan) 55 towards tumor cells has been



55

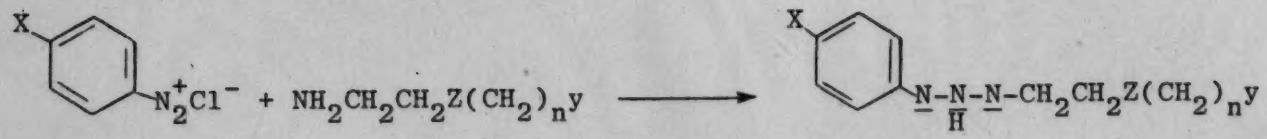
attributed to the concentration of phosphomidase[99] in tumor cells that liberates nitrogen mustard. Sulfur mustard as such is too reactive for clinical use. The concept of using triazenes as a transport form of sulfur mustard is based on the possibility of a selective decomposition of

monoalkyltriazenes in neoplastic tissue due to slightly lower pH than the pH exhibited by the normal tissue.

Studies on the Synthesis and Decomposition of 1-phenyl-3-{S-(2-chloroethyl)thioethyltriazenes and Related Compounds

The triazenes were prepared by coupling of the corresponding aryl diazonium cations with appropriate primary amines (Table 10). The electron-withdrawing groups were maintained in the phenyl ring for stability as well as for ease of purification of the triazenes as discussed in Chapter II. The general behaviour of the triazeno-function of this series of triazenes showed a marked similarity to that of other monoalkyltriazenes reported earlier. For the selected compound 56 the rate of decomposition in aqueous medium determined polarographically increases markedly with decreasing pH in the range 5.1 to 7.1 (Table 11). The triazenes were unstable on solid adsorbents thus preventing their purification by standard chromatographic method. Instead they were recrystallized at low temperatures. The composition, structures and purity of the triazenes were established by exact mass spectral measurements and by pmr and ir spectroscopy. Esterification of 3,5-dinitrobenzoic acid with these triazenes provided additional structural proof. The triazenes of this series were found to be thermally as well as chemically more stable than the corresponding 2-chloroethyltriazenes reported in

TABLE 10



X	y	z	n	Compound	
p-CN	Cl	S	2	1-(p-cyanophenyl)-3-{S-(2-chloroethyl)thioethyl}triazene	56
p-$COCH_3$	Cl	S	2	1-(p-acetylphenyl)-3-{S-(2-chloroethyl)thioethyl}triazene	57
p-$COOC_2H_5$	Cl	S	2	1-(p-ethoxycarbonylphenyl)-3-{S-(2-chloroethyl)thioethyl}triazene	58
p-NO_2	Cl	S	2	1-(p-nitrophenyl)-3-{S-(2-chloroethyl)thioethyl}triazene	59
p-CN	Cl	S	3	1-(p-cyanophenyl)-3-{S-(3-chloropropyl)thioethyl}triazene	62
p-CN	Cl	O	2	1-(p-cyanophenyl)-3-{O-(2-chloroethyl)ethoxy triazene}	63
p-CN	OH	S	2	1-(p-cyanophenyl)-3-{S-(2-hydroxyethyl)thioethyl}triazene	64

1-(p-cyanophenyl)-3-{S-(2-chloroethyl) 1,1-dideuteriothioethyl}triazene 67

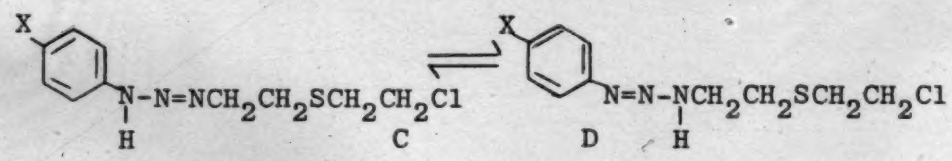
TABLE 11

VARIATION OF HALF-LIFE ($t_{\frac{1}{2}}$) OF THE TRIAZENE 56 DETERMINED POLAROGRAPHICALLY AS A FUNCTION OF SOLUTION pH at 25°

pH	$t_{\frac{1}{2}}$ (sec)
5.1	156
5.8	300
7.1	1362

Chapter II.

As observed for monoalkyltriazenes in general, S-(2-chloroethyl)thioethyltriazenes exist as a mixture of tautomers in solution. An electron-withdrawing group X in the *para* position strongly favours the tautomeric form C. However, triazenes with an electron-withdrawing group



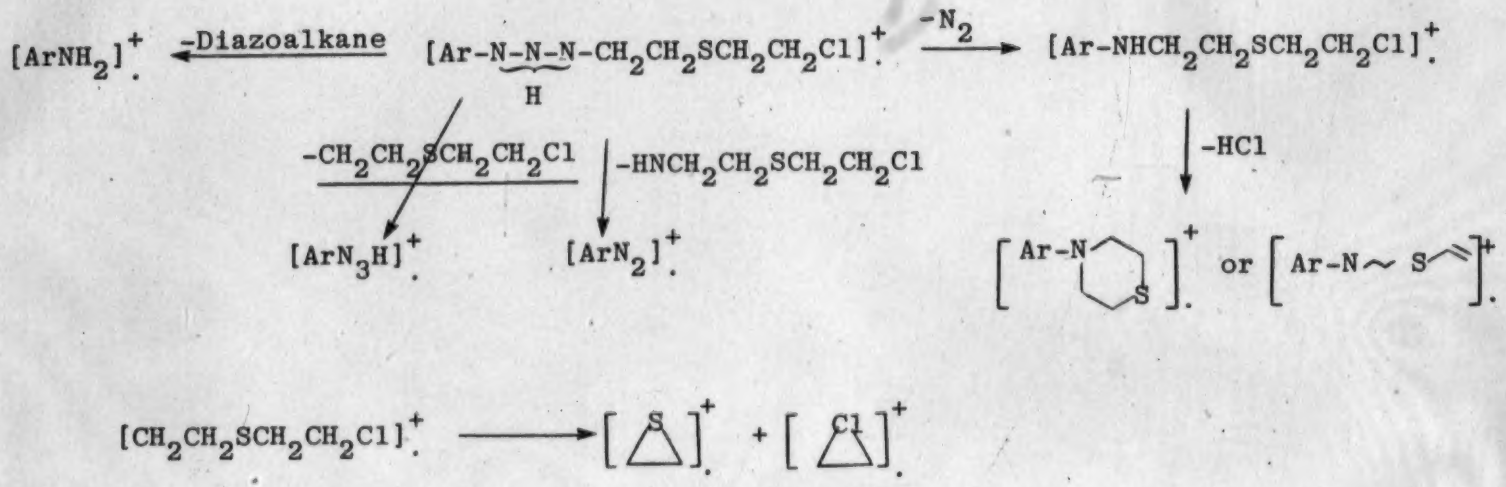
X = NO_2, CN, $COCH_3$, $CO_2C_2H_5$

in the *ortho* position are unstable, plausibly for the reasons given earlier (see Chapter II).

The mass spectra of the S-(2-chloroethyl)thioethyltriazenes show the general fragmentation pattern outlined for 2-haloalkyltriazenes in Chapter II. Additionally, they show a characteristic high relative intensity peak due to the intact side chain which further fragments to give ions corresponding to episulfide and 2-chloroethyl group, again, in relatively high intensities. Another common feature of these molecules is sulfur assisted elimination of HCl which occurs after the loss of nitrogen. This is not observed when sulfur is replaced by oxygen, or if there are three methylene units intervening between sulfur and chlorine (Scheme 13).

Triazene 67 specifically deuterated on C-1 was synthesized from the corresponding deuterated amine in order to

SCHEME 13


[ArNH2]+.
-Diazoalkane
[Ar-N-N-N-CH2CH2SCH2CH2Cl]+.
H
-N2
[Ar-NHCH2CH2SCH2CH2Cl]+.
-CH2CH2SCH2CH2Cl
-HNCH2CH2SCH2CH2Cl
-HCl
[ArN3H]+.
[ArN2]+.
Ar-N
S
or
Ar-N
S

[CH2CH2SCH2CH2Cl]+.
S
Cl

establish the mechanistic pathways of formation of certain products. Triazenes 62 and 63 were synthesized in order to study the involvement of sulfur in determining the chemical reactivity of S-(2-chloroethyl)thioethyltriazenes in general.

Studies Related to the Decomposition of Triazenes

The aqueous chemistry of the triazenes with sulfur activated side chain was studied relative to the similar triazenes incorporating structural features designed to deactivate the side chain. The use of specific deuterium labelling in the activated side chain was also informative.

Five triazenes were allowed to decompose at 37° in phosphate buffered (0.1 M, pH 7.2) aqueous solution in gastight vials and the volatile products were analyzed by GC and identified by GC/MS (Table 12). The involatile products were separated by chromatography and identified spectrophotometrically. 1-(p-cyanophenyl)-3-{S-(2-chloroethyl)thioethyl}triazene 56 afforded bis(2-chloroethyl)-sulfide 68 (2% of volatiles) and bis(2-hydroxyethyl)sulfide 69 (72% of volatiles). In addition the following involatile products were identified: p-aminobenzonitrile 70 60% of involatiles); N-{S-(2-chloroethyl)thioethyl}-p-cyanoaniline 71 (4%) and N-{S-(2-hydroxyethyl)thioethyl}-p-cyanoaniline 72 (16%) (Scheme 14).

Aqueous decomposition of the corresponding specifically deuterium labelled triazene 1-(p-cyanophenyl)-3-

TABLE 12

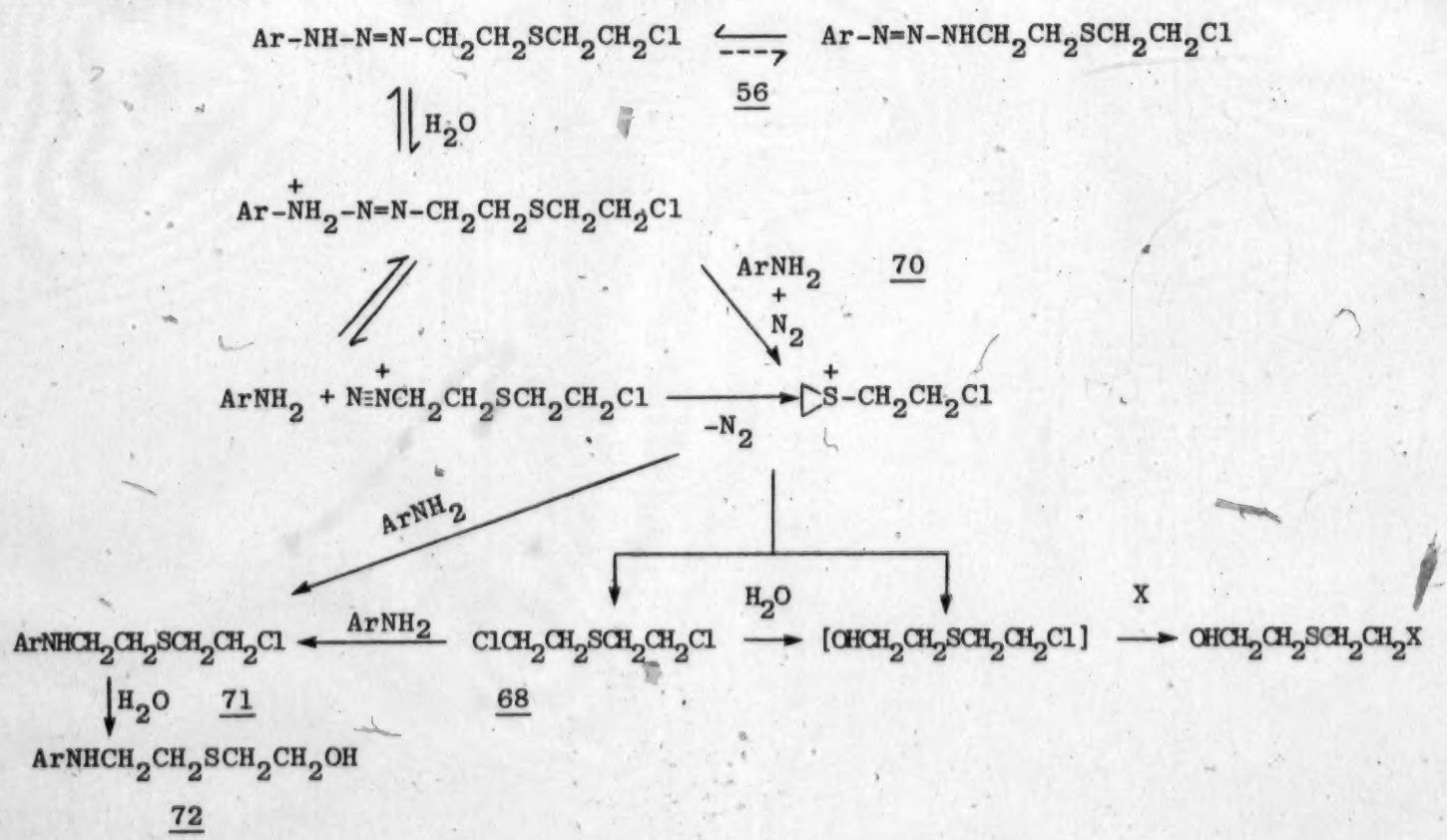
MASS SPECTRAL IDENTIFICATION OF PRODUCTS OF AQUEOUS AND NON-AQUEOUS DECOMPOSITION OF PROTIUM AND SPECIFICALLY DEUTERIUM LABELLED S-(2-CHLOROETHYL)THIOETHYTRIAZENES AND RELATED COMPOUNDS

Triazene	Reaction Conditions	Decomposition Products	m/e (Relative Intensity, fragments)
56	phosphate buffer	$ClCH_2CH_2SCH_2CH_2Cl$	158 (21.6, M^+); 160 (15.0, M^+, Cl^{37}); 162 (3.1, M^+ 2 Cl^{37}); 109 (100, CH_2S-CH_2CH_2Cl)
56	anhydrous DME	$ClCH_2CH_2SCH_2CH_2Cl$ + $CH_2=CHSCH_2CH_2Cl$ ⟶	122 (38.2, M^+); 124 (13.7, M^+, Cl^{37})
67	phosphate buffer	$ClCD_2CH_2SCH_2CH_2Cl$ + $ClCH_2CD_2SCH_2CH_2Cl$	160 (22.6, M^+); 162 (15.2, M^+, Cl^{37}); 164 (2.7, M^+, 2 Cl^{37}); 109 (30.7, $CH_2SCH_2CH_2Cl$); 111 (100, $CD_2SCH_2CH_2Cl$); 113 (32.5, $CD_2SCH_2CH_2Cl^{37}$)
62	phosphate buffer	$CH_2=CH-S-CH_2CH_2CH_2Cl$	136 (26.2, M^+); 138 (9.4, M^+, Cl^{37}); 101 (6.4, $CH_2=CHS-CH_2CH_2-CH_2$); 60 (100, $CH_2=CH-S$)
		$ClCH_2CH_2SCH_2CH_2CH_2Cl$	172 (24.6, M^+); 174 (17.8, M^+, Cl^{37}); 176 (3.2, M^+, 2 Cl^{37}); 123 (60.9, CH_2S-$CH_2CH_2CH_2Cl$); 125 (22.2, $CH_2SCH_2CH_2CH_2$-Cl^{37})

Table 12, continued

Triazene	Reaction Conditions	Decomposition Products	m/e (Relative Intensity, fragments)
63	phosphate buffer	$CH_2=CH-O-CH_2CH_2Cl$	106 (21.6, M^+); 108 (6.8, M^+, ^{37}Cl); 63 (100, CH_2CH_2Cl)
		$ClCH_2CH_2O-CH_2CH_2Cl$	142 (0.9, M^+); 144 (0.5, M^+, ^{37}Cl); 93 (100, $CH_2-O-CH_2CH_2Cl$); 95 (31.4, $CH_2-O-CH_2CH_2Cl^{37}$)

SCHEME 14

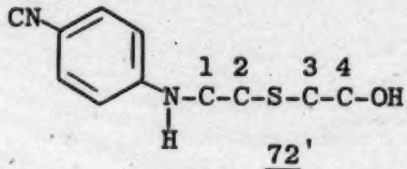


Ar = p-CNC_6H_4

X = OH or p-CNC_6H_4NH

69

{S-(2-chloroethyl)-1,1-dideuteriothioethyltriazene 67 afforded N-{S-(2-hydroxyethyl)thioethyl}-p-cyanophenylaniline 72' with deuterium on all four carbons in the side chain. The percent distribution of deuterium on carbon 1, 2, 3 and 4 was approximately 15, 15, 33 and 37 respectively.

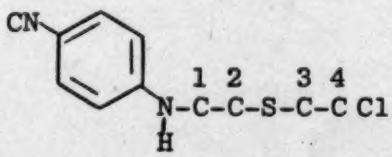


72'

[H and D around C are not shown]

The deuterated product 71' corresponding to 71 had deuterium on carbon bearing chlorine predominantly.

When triazene 67 was decomposed in anhydrous 1,2-dimethoxyethane the involatile product 71' was formed in a low yield (5%) with deuterium on C_1, C_2 and C_4 in approximately equal amounts. There was no detectable amount of deuterium on C_3 in 71'. The aromatic amine 70 was the

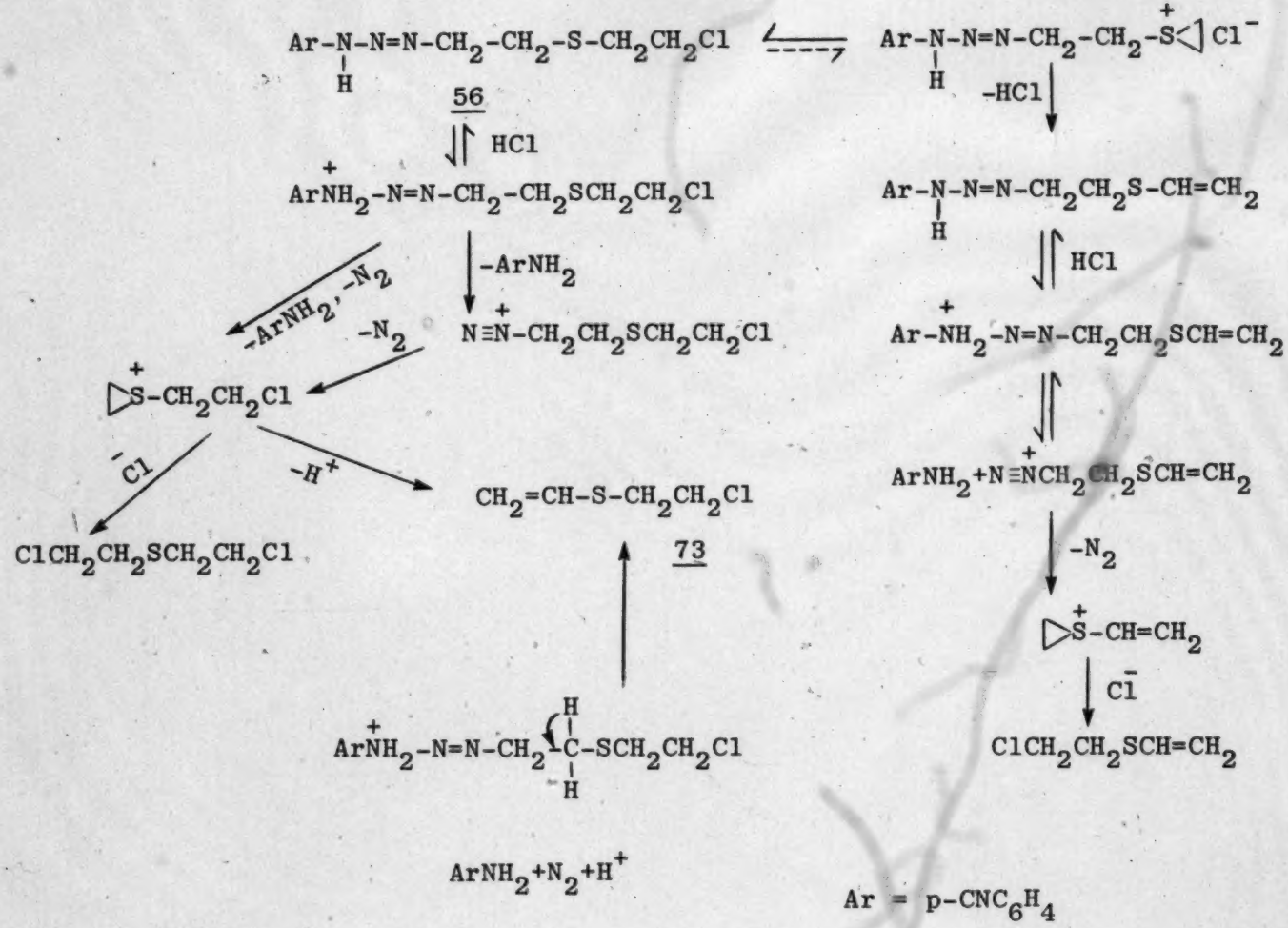


71'

[H and D around C are not shown]

major product. The volatile products obtained from non-aqueous decomposition of 56 contained chloroethylvinyl sulfide 73 and bis(β-chloroethyl)sulfide 68 in the ratio 1:2 (Scheme 15).

SCHEME 15

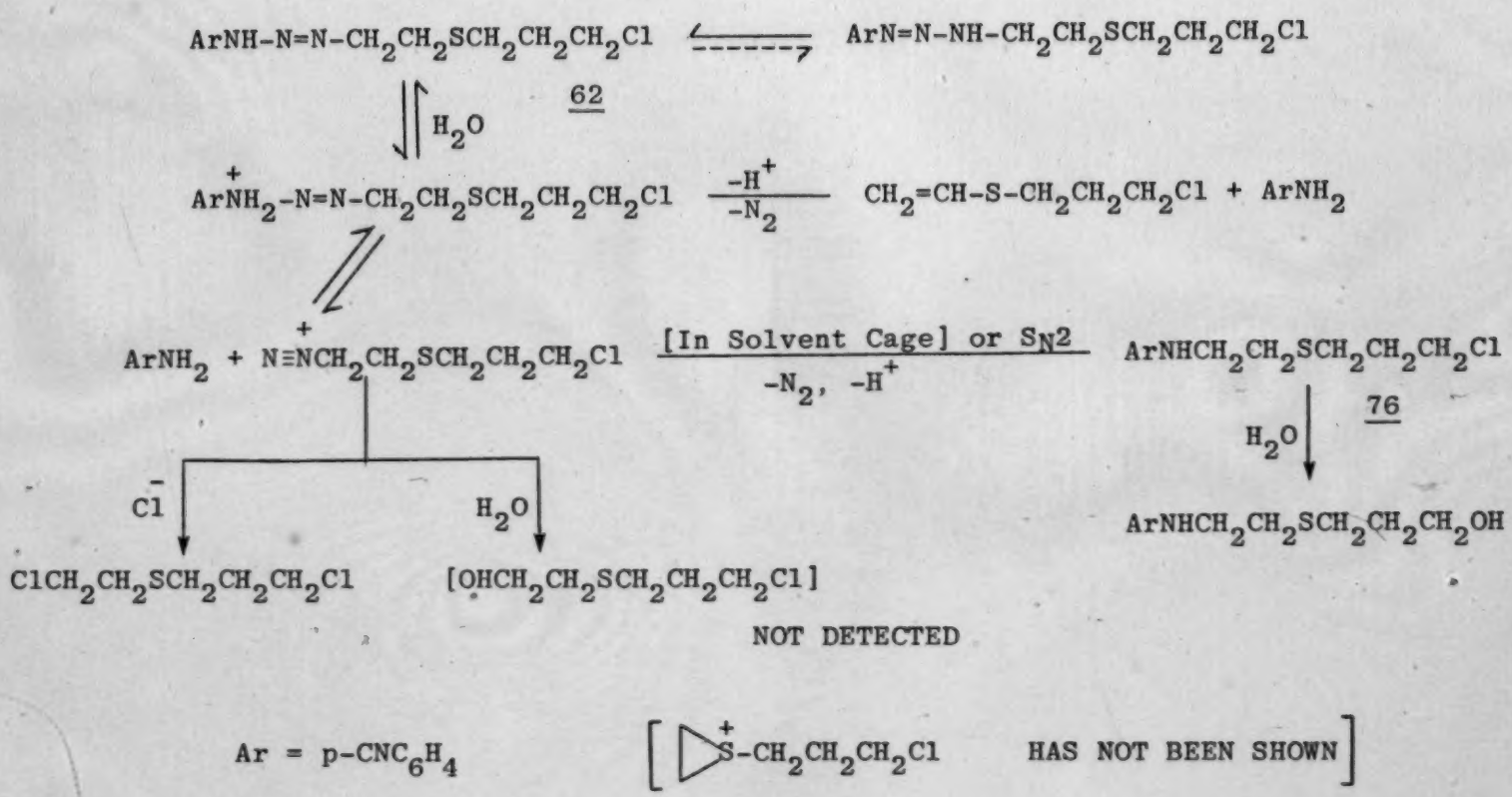

Ar-N(H)-N=N-CH2-CH2-S-CH2CH2Cl
56
HCl
ArNH2-N=N-CH2-CH2SCH2CH2Cl
-ArNH2
-ArNH2, -N2
-N2
N≡N-CH2CH2SCH2CH2Cl
S-CH2CH2Cl
-Cl
-H+
ClCH2CH2SCH2CH2Cl
CH2=CH-S-CH2CH2Cl
73
ArNH2-N=N-CH2-C(H)(H)-SCH2CH2Cl
ArNH2+N2+H+
Ar-N(H)-N=N-CH2-CH2-S+ Cl-
-HCl
Ar-N(H)-N=N-CH2CH2S-CH=CH2
HCl
Ar-NH2-N=N-CH2CH2SCH=CH2
ArNH2+N≡NCH2CH2SCH=CH2
-N2
S-CH=CH2
Cl-
ClCH2CH2SCH=CH2
Ar = p-CNC6H4

All involatile products obtained from aqueous as well as non aqueous decompositions of 67 showed retention of both deuteriums.

Aqueous decomposition of 1-(p-cyanophenyl)-3-{S-(3-chloropropyl)thioethyl}triazene 62 afforded 3-chloropropyl vinyl sulfide 74 and 2-chloroethyl, 3-chloropropyl sulfide 75 in the ratio (3:2) accounting for ~8% of the volatiles. The involatile products N-{S-(3-chloropropyl)thioethyl}-p-cyanoaniline 76 and N-{S-(3-hydroxypropyl}thioethyl-p-cyanoaniline 77 together accounted for ~3% of the involatiles and p-aminobenzonitrile 70 was the major product (Scheme 16). From the aqueous decomposition of 1-(p-cyanophenyl)-3-{O-(2-chloroethyl)ethoxy}triazene 63, 2-chloroethylvinylether 78 and bis(2-chloroethyl)ether 79 in the ratio 2:1 (9% of the total volatiles) were identified as the volatile products. Involatile products N-{O-(2-chloroethyl)ethoxy}-p-cyanoaniline 80 and N-{O-(2-hydroxyethyl)-ethoxy}-p-cyanoaniline 81 together accounted for less than 5% of the involatiles and p-aminobenzonitrile 70 was the major product (Scheme 17). Triazene 64 on decomposition afforded N-{S-(2-hydroxyethyl)thioethyl}-p-cyanoaniline 72 in 8% yield in addition to p-aminobenzonitrile 70 as the major involatile product (Scheme 18).

When bis-(2-chloroethyl)sulfide 68 was allowed to react with p-aminobenzonitrile in aqueous ethanol the major products were bis-{2-(p-cyanoanilino)ethyl}sulfide 82 and 72 formed in the ratio 4:1 respectively in 72% yield. The

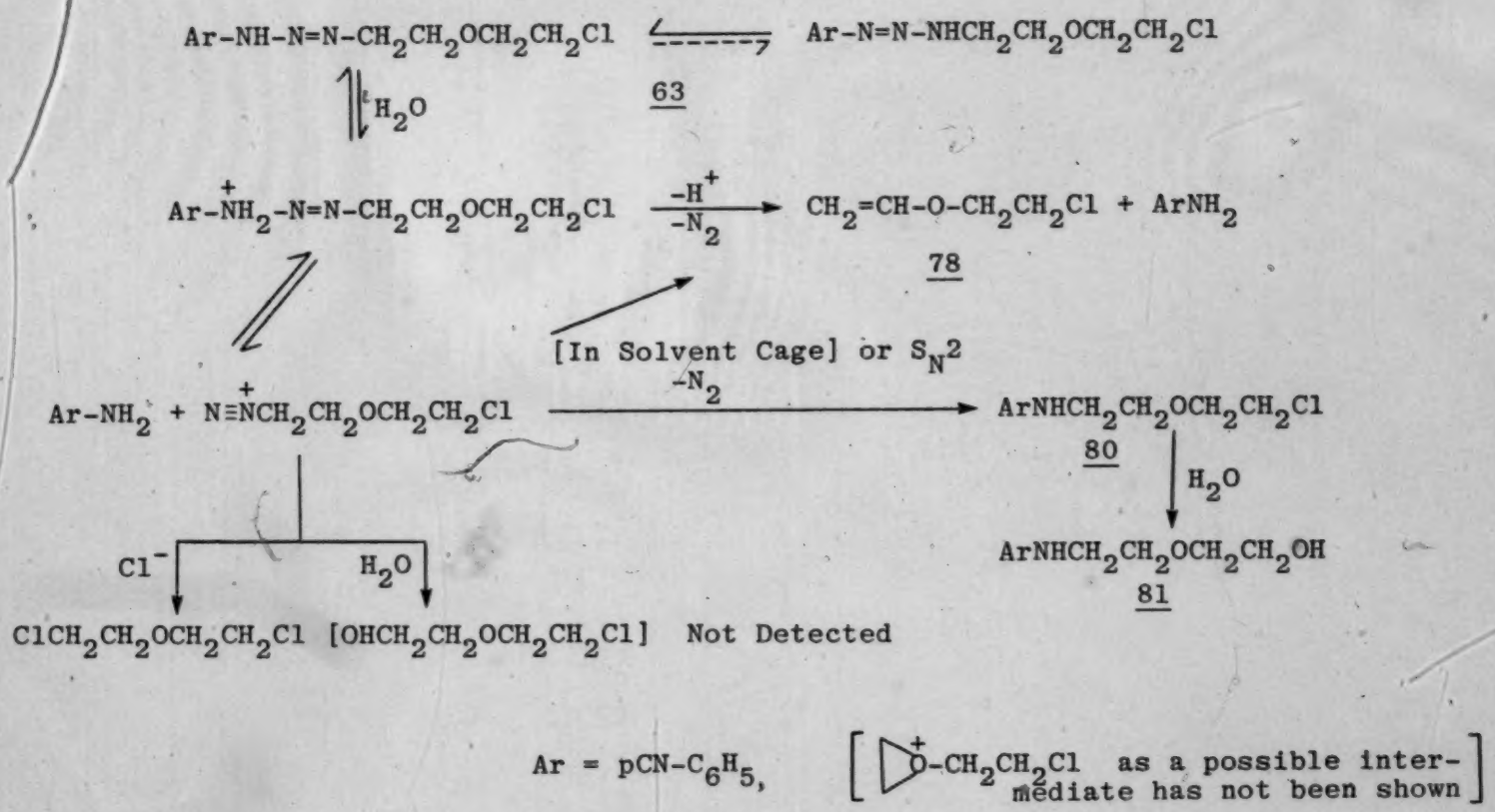
SCHEME 16



Ar = p-CNC_6H_4

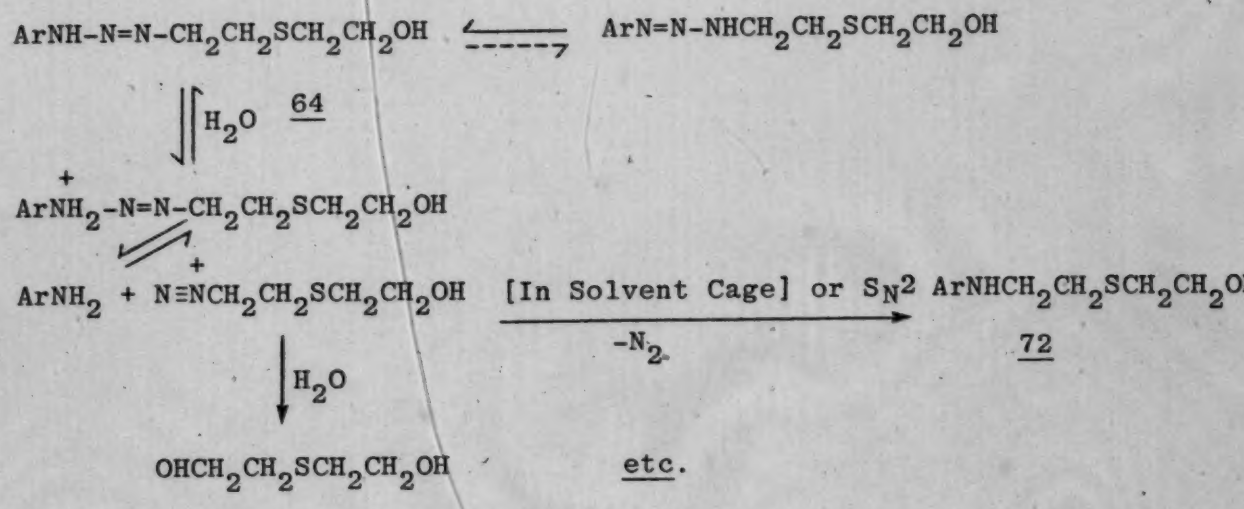
$\left[\text{ArS}^{+}\text{-}CH_2CH_2CH_2Cl\right]$ HAS NOT BEEN SHOWN

SCHEME 17



Ar = pCN-C_6H_5, [as a possible intermediate has not been shown]

SCHEME 18


ArNH-N=N-CH2CH2SCH2CH2OH
ArN=N-NHCH2CH2SCH2CH2OH
H2O
64
+
ArNH2-N=N-CH2CH2SCH2CH2OH
+
ArNH2 + N≡NCH2CH2SCH2CH2OH
[In Solvent Cage] or SN2
-N2
ArNHCH2CH2SCH2CH2OH
72
H2O
OHCH2CH2SCH2CH2OH
etc.

aniline derivative 71 was a minor product [Equations 1, 2, 3].

$$ArNH_2 + ClCH_2CH_2SCH_2CH_2Cl \longrightarrow \underset{\underline{71}}{ArNHCH_2CH_2SCH_2CH_2Cl} + HCl \quad [1]$$

$$ArNHCH_2CH_2SCH_2CH_2Cl + H_2O \longrightarrow \underset{\underline{72}}{ArNHCH_2CH_2SCH_2CH_2OH} + HCl \quad [2]$$

$$ArNHCH_2CH_2SCH_2CH_2Cl + ArNH_2 \longrightarrow \underset{\underline{82}}{ArNHCH_2CH_2SCH_2CH_2NHAr} + HCl \quad [3]$$

The same reaction in anhydrous 1,2-dimethoxyethane afforded 82 and 71 in approximately equal amounts in 40% yield. The aniline derivative 71 hydrolyzed to 72 under the conditions used for the aqueous decompositions of the triazenes 56 and 67.

The observed dependence of the rate of decomposition of the present series of triazenes on the pH of the medium parallels our previous observations with 2-haloalkyltriazenes suggesting that a similar mechanism could be operative at least in the initial stages of decomposition. The protonation of the major tautomer is followed by either cleavage of the N_1-N_2 bond of the triazene or the protonated triazene becomes susceptible to S_N2 attack by electrophiles at the carbon-bonded to N_3 in the side chain (Schemes 14 and 15). Sulfur assisted hydrolysis of the chloride in the side chain to form the corresponding 3-S-(2-hydroxyethyl)thioethyltriazene 64 prior to the decomposition of the triazeno-function could be a competing route as indicated by the decomposition product N-{S-(2-hydroxyethyl)-

thioethyl-p-cyanoaniline 72 obtained from 64.

A number of alternative pathways may be considered to interpret the observed distribution of deuterium labels in the products. An episulfonium species may plausibly account for the scrambling of deuterium between C_1 and C_2. The sulfur assisted hydrolysis of the half mustard is facile since the polar solvent promotes ionization.

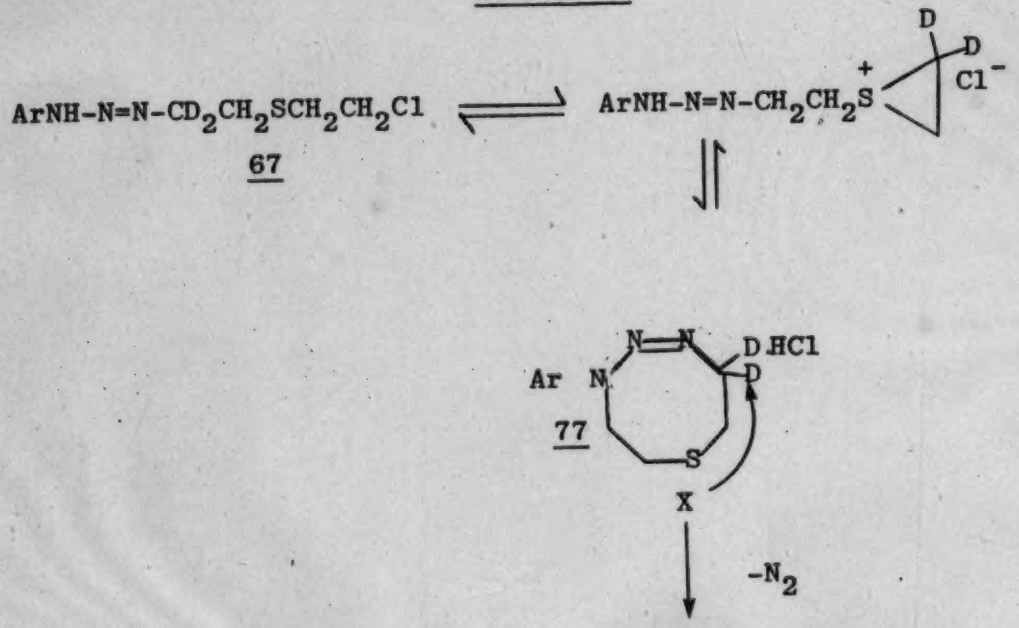
The formation of cyclic intermediate 77 may account for the localization of deuterium on C_3 and C_4 in the side chain of the involatile products from 66. Precedence for such an intermediate exists in the case of 2-haloalkyl triazenes (Scheme 19). The sulfur assisted labilization of carbon-chlorine bond in the side chain of the triazene promoting the cyclization to form the triazathiaoctene may overcome the entropic factor involved. If formed the cyclic species 77 correctly predicts the scrambling pattern.

From our experience with 2-chloroethyltriazene 16 the major product initially generated from the triazathiaoctene ion-pair should be 71' with deuterium localized on carbon bearing chlorine and the minor product should be 72' with deuterium on carbon bearing hydroxyl group.

If the deuterated derivative 71' hydrolyses through an episulfonium intermediate the deuterium has an equal probability of localization on C_3 and C_4. Therefore the amount of deuterium on C_4 will be greater than the amount on C_3.

However, an alternative explanation that can account for the biased accumulation of deuterium on C-4 in 72' is

SCHEME 19



Ar = pCN C_6H_4

the possibly predominant generation of β-chloroethyl, β'-hydroxyethyl sulfide directly from the triazene 67 via S_N2 process (see the esterification of the carboxylic acid) that will direct the localization of deuterium on carbon bearing the hydroxyl group. The sulfur half-mustard portion of this species can proceed to alkylate the amine without further scrambling of deuterium. Structurally related bis-(β-chloroethyl)sulfide alkylates p-aminobenzonitrile.

The greatly reduced yield of 71' formed from the non-aqueous decomposition of 67 indicated the inability of the solvent to sustain charged intermediates and hence the generation of episulfonium intermediates is retarded. This in turn could be reflected in the retardations of sulfur assisted intramolecular cyclization, intermolecular alkylation of the amine by episulfonium species and the scrambling of deuteriums on C_4 in 71'.

Owing to the multiplicity of the mechanistic possibilities for the formation of the aniline derivatives from the triazene 56 it is not possible to delineate a unique mechanism at this time. However, in view of the substantially higher yields of the involatile products from 56 compared with those from 62 and 63 it may be suggested that the alkylation of amine in the decomposition of 56 proceeds predominantly intramolecularly.

2-Chloroethylvinylsulfide could not be detected in the aqueous decomposition of 56 although 3-chloropropylvinyl-

sulfide 74 and 2-chloroethylvinylether 78 accounted for a substantial portion of the volatile products from 62 and 63 respectively. A somewhat similar observation was made in our study with 2-chloroethyltriazene where vinylchloride could not be detected in its aqueous decomposition whereas 2-chloropropyltriazene gave 2-chloropropene (Scheme 7). Whether this observation indicates that the elimination products are obtained mainly from the conjugate acids of the triazenes in a slow process in the absence of intramolecular cyclization, can not be said with certainty.

The alcohols which were anticipated among the volatile products could not be detected from 62 and 63 under our analytical conditions.

Compound 76 is the only chloroalkylamine obtained from 62. Alkylation of aniline by the decomposition product 2-chloroethyl,3-chloropropylsulfide 75 presumably proceeds with the preferential activation of the 2-chloroethyl moiety.

Uridine is of low reactivity toward most alkylating agents except diazoalkanes.[73] An earlier attempt[100] to study the alkylation of the structurally related nucleoside thymidine with sulfur mustard did not lead to any identifiable product.

When the triazene 56 was allowed to react with triacetyl uridine in anhydrous 1,2-dimethoxyethane the N^3-alkylated product 83 was obtained in 8% yield. On using the specifically deuterated triazene 67 for alkylation the corres-

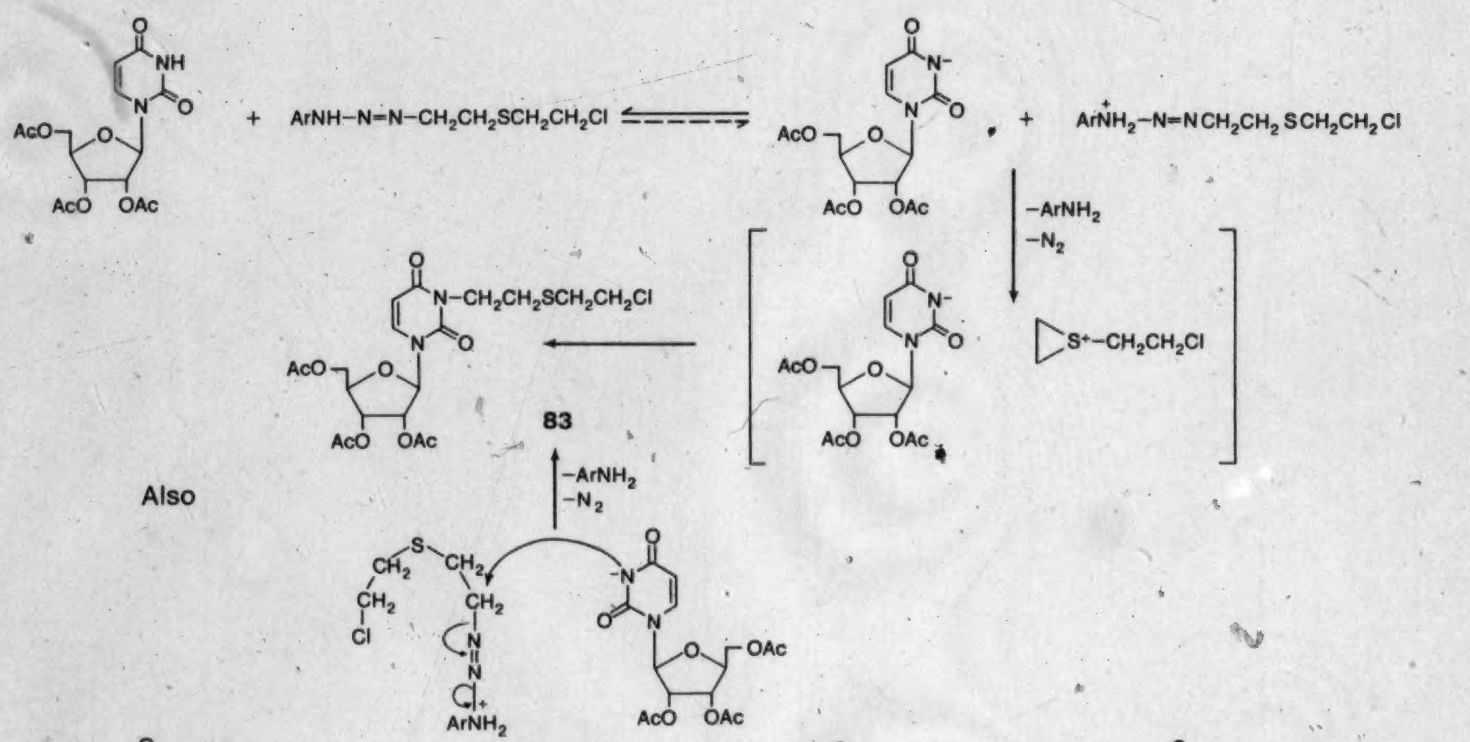
ponding product 83' contained deuterium in the side chain on carbons α and β to N^3 in a ratio 3:2. The observations indicate that N^3-proton of uridine with a pK_a of 9.17 can protonate the triazene, most likely at N-1 position followed by its decomposition generating episulfonium alkylating species and the alkylation proceeds via an ion-pair mechanism (Scheme 20). However, the relative distribution of deuterium in the side-chain of the product 83' indicates a certain degree of S_N2-displacement by the pyrimidine nitrogen in a fashion similar to the one discussed subsequently.

Mechanism of Esterification of Carboxylic Acids by Monoalkyltriazenes

The triazenes of this series also readily esterify 3,5-dinitrobenzoic acid and diethylphosphate as observed for 2-haloalkyltriazenes discussed in previous chapters. The stability of the alkylcarbonium ion generated from 3,5-(2-chloroethyl)thioethyltriazenes is greatly influenced by the presence of sulfur at the β-position. This may be contrasted with the effect of chlorine on the stability of the 2-chloroethyl cation via chloronium intermediate, generated from 2-chloroethyltriazenes. This feature of the former class of triazenes provided a further opportunity to examine the mechanism of esterification by monoalkyltriazenes.

A previous study[58] of this reaction was based on

Scheme 20

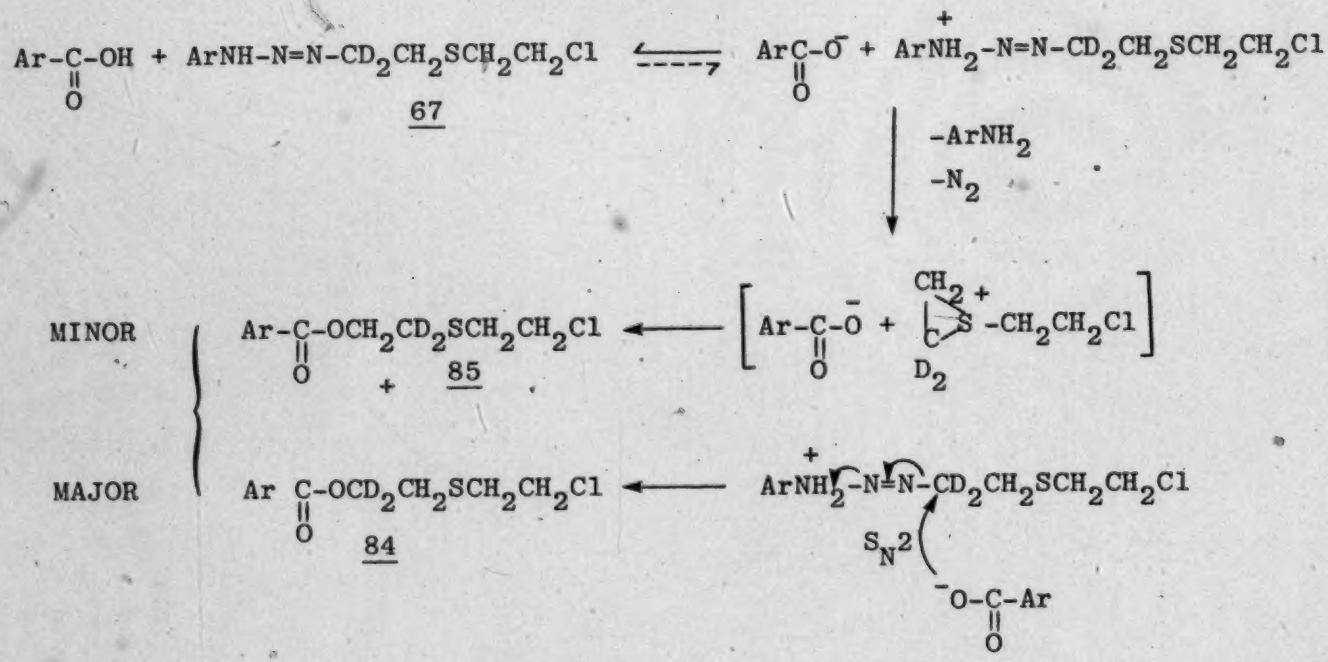

ArNH–N=N–CH2CH2SCH2CH2Cl
ArNH2–N=NCH2CH2SCH2CH2Cl
–ArNH2
–N2
83
Also
ArNH2
ArNH–N=N–CD2CH2SCH2CH2Cl
67
83′
+ ArNH2 + N2

kinetic measurements of the rate of decomposition of monoalkyltriazenes by substituted benzoic acids as a function of alkyl group substitution. This study led to the conclusion that the alkyl group leaves as a cation in a rate-determining step. The observed rate order Me<Et<Pr^i<Bu^t corresponded to increasing stabilization of a cation and the reverse order to that expected for a bimolecular substitution at carbon. However, altering the degree of substitution in the alkyl group also alters steric factors that could influence the course of the reaction.

The present investigation employed a combination of specifically deuterated triazenes 33 and 67 in which the relative stabilities of the potential alkyl carbonium ions have been altered while keeping the steric parameter constant. When triazene 67 was allowed to react with 3,5-dinitrobenzoic acid the product was a mixture of S-(2-chloroethyl)-1,1-dideuteriothioethyl, 3,5-dinitrobenzoate 84 and S-(2-chloroethyl)-2,2-dideuteriothioethyl-3,5-dinitrobenzoate 85 formed in the ratio 60:40.

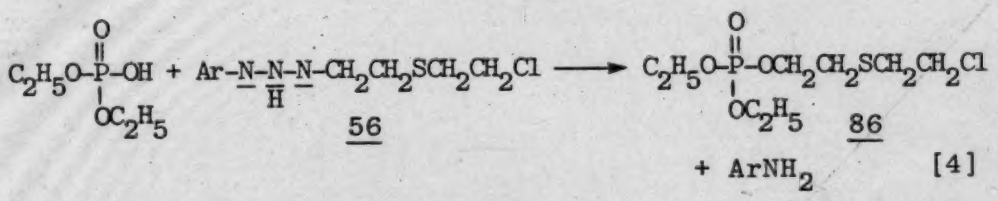
The distribution of deuterium between the esters 84 and 85 indicates S_N2 displacement as a minor pathway and that the stability of alkylating episulfonium species contributes largely to an ion-pair mechanism (Scheme 21). In contrast the less stable chloronium ion that would be generated from the 2-chloroethyltriazene 33 does not contribute to any ion-pair mechanism and the esterification proceeds exclusively via S_N2 displacement as discussed in Chapter II.

SCHEME 21


Ar-C(=O)-OH + ArNH-N=N-CD2CH2SCH2CH2Cl
67
ArC(=O)-O⁻ + ArNH2⁺-N=N-CD2CH2SCH2CH2Cl
-ArNH2
-N2
MINOR
MAJOR
Ar-C(=O)-OCH2CD2SCH2CH2Cl + 85
Ar C(=O)-OCD2CH2SCH2CH2Cl
84
CH2
S -CH2CH2Cl
D2
SN2
⁻O-C(=O)-Ar

It appears that the mechanism of the esterification depends on the nature of the alkylating species. The increasing steric hindrance at the site of reaction in the triazene and the formation of stable carbonium ions contribute increasingly to the ion-pair mechanism.

S-(2-chloroethyl)thioethyltriazenes readily esterify diethyl phosphate as observed with 2-haloalkyltriazenes. Triazene 56 gave diethyl,S-(2-chloroethyl)thioethyl phosphate 86 in 10%yield when allowed to react with diethyl phosphate in anhydrous ether [Equation 4].



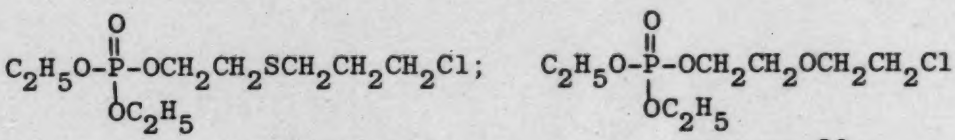
[4]

It is conceivable that triazenes may alkylate acidic sites on DNA because of their unique acid catalyzed decomposition. However, irrespective of the site of initial alkylation on DNA the sulfur half mustard portion from the alkylated sites can proceed to alkylate various sites on the complementary strand resulting in interstrand cross-links. This has been verified in the subsequent chapter using DNA.

γ-(p-nitrobenzyl)pyridine (NBP) has been used for qualitative detection as well as quantitative estimation of various alkylating species including nitrogen and sulfur mustards.[101] The method depends upon the capacity of a reagent to alkylate NBP and thereby convert it to the quaternary pyridinium state which is highly colored in an

alkaline medium (Scheme 22). In a typical reaction, a mixture of an alkylating reagent and NBP is heated for a standard period of time (20 min). After cooling and introduction of alkali the intensity of the color developed is measured spectrophotometrically at 600 nm. Absolute concentrations can be determined using a standard curve. This method was used to compare the alkylating properties of the triazenes employed in the DNA cross-linking study.

The phosphate triesters 87 and 88 prepared from the

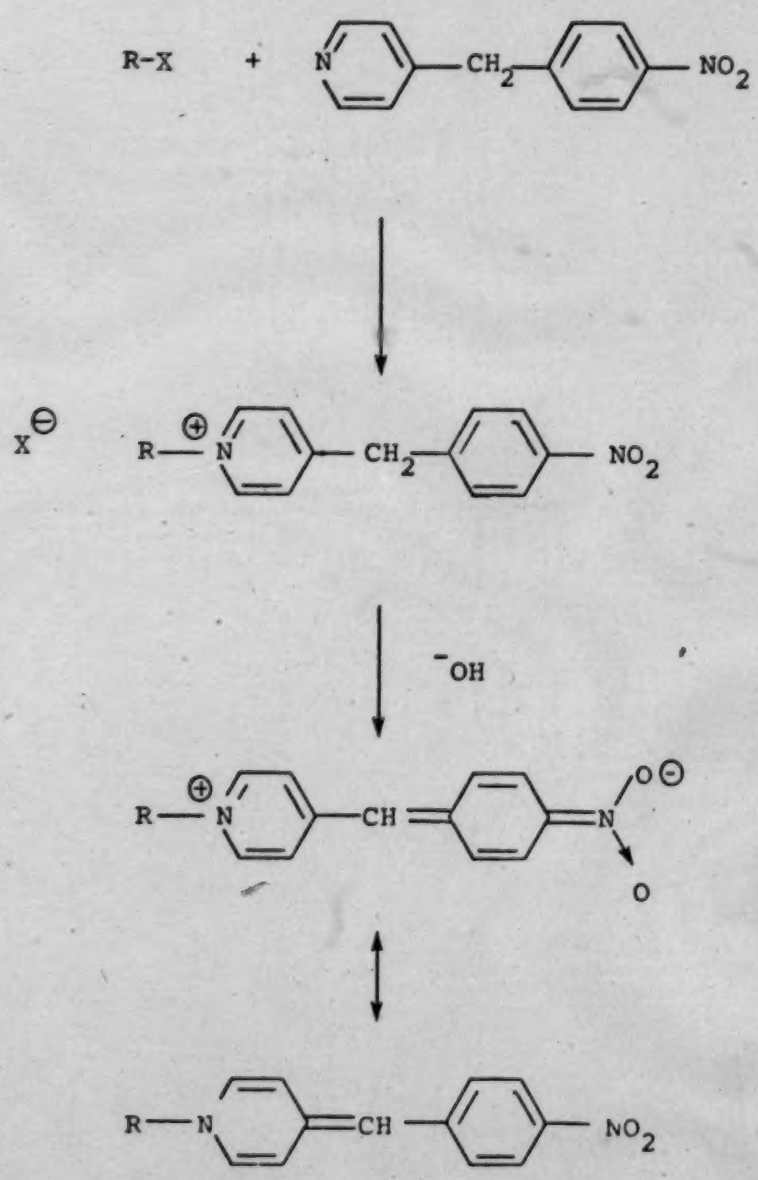


87

88

triazenes 62 and 63, respectively, along with the uridine derivative 83, and the phosphate triester 86, prepared from the triazene 56, were allowed to react with NBP under similar conditions. Compounds 83 and 86 alkylate NBP while 87 and 88 failed to show any sign of alkylation. It is conceivable that in 83 and 86 the presence of sulfur in the side chain promotes alkylation through easily formed episulfonium intermediates whereas the phosphotriesters 87 and 88, lacking such activation, fail to alkylate NBP.

SCHEME 22



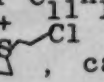
EXPERIMENTAL

1(3)-Aryl-3(1)-{S-(2-chloroethyl)thioethyl}triazenes

General Procedure

The aromatic amine (10 mmol) was diazotized following the procedure given in Chapter II. The neutralized diazonium salt solution was allowed to react with S-(2-chloroethyl)thioethylamine free base[102] (10 mmol) at -5° by stirring for 5-10 min. Usually the triazene precipitated as a solid that was purified by recrystallization from methylene chloride/petroleum ether mixtures at low temperatures.

The general procedure afforded the following triazenes

1-(p-Cyanophenyl)-3-{S-(2-chloroethyl)thioethyl}triazene 56 (1.4 g, 52%), m.p. 72-73°; ν_{max} 3182.5, 3158.9, 2222, 1609 cm^{-1} δ ($CDCl_3$, -45°) 2.9 (m, 4H, CH_2-S-CH_2), 3.7 (t, 2H, CH_2-Cl), 4.0 (t, 2H, N-CH_2), 7.15 - 7.75 (m, 4H, aromatic), 8.75 and 9.7 {t and s (1:5), 1H, exchangeable, N-H}; M^+ 268.0545 (1.30%) (calcd. for $C_{11}H_{13}ClN_4S$ 268.0550), 240.0485 (2.0%) (M-N_2, calcd. for $C_{11}H_{13}ClN_2S$, 240.0488), 204.0715 (0.38%) (M-N_2)-HCl, calcd. for $C_{11}H_{12}N_2S$, 204.0721) 118.0533 (100%); 123.0035 (19.12%); ( , calcd. for C_4H_8ClS, 123.0035). Anal. calcd. for $C_{11}H_{13}ClN_4S$: C, 49.16, H = 4.84, Cl = 13.22, N = 20.85 S = 11.92. Found: C = 48.98, H = 4.81, Cl = 13.41, N = 21.20, S = 11.60.

1-(p-Acetylphenyl)-3-{S-(2-chloroethyl)thioethyl)} triazene 57

(.8 g, 29%), m.p. 64°, ν_{max} 3220, 3193 and 1600 cm^{-1}. δ (CD_2Cl_2, 30°) 2.5 (s, 3H, Me), 2.9 (t, 4H, CH_2-S-CH_2), 3.65 (t, 2H, CH_2-Cl), 3.9 (t, 2H, N-CH_2), 7.15 - 8.0 (m, 4H, aromatic), 9.2 (broad, s, exchangeable, 1H, N-H), M^+ 285.0695 (0.60%), (calcd. for $C_{12}H_{16}ClN_3OS$, 285.0703), 257.0638 (3.41%), (M-N_2, calcd. for $C_{12}H_{16}ClNOS$, 257.0641), 221.0871 (0.89%), ((M-N_2)-HCl, calcd. for $C_{12}H_{15}NOS$, 221.0874), 135.0682 (64.34%) 120.0452 (100%); 123.0037 (13.38%) (, calcd. for C_4H_8ClS, 123.0035).

1-(p-Ethoxycarbonylphenyl)-3-{S-(2-chloroethyl)thioethyl} triazene 58

(0.90 g, 29%), m.p. 54-56°, ν_{max} 3250.9, 1687.3, 1605.8; δ ($CDCl_3$, -15°) 1.4 (t, 3H, Me), 2.90 (t, 4H, CH_2-S-CH_2), 3.7 (t, 2H, CH_2-Cl), 3.95 (t, 2H, N-CH_2), 4.35 (q, 2H, O-CH_2), 7.1 - 8.2 (m, 4H, aromatic), 8.5 and 9.5 (br, 2s, 1H, exchangeable, N-H); M^+ 315.0804 (3.02%) (calcd. for $C_{13}H_{18}ClN_2O_2S$, 315.0808), 287.0746 (13.66%) (M-N_2, calcd. for $C_{13}H_{18}ClNO_2S$, 287.0746), 251.0983 (3.39%) ((M-N_2)-HCl, calcd. for $C_{13}H_{17}NO_2S$, 251.0980), 165.0785 (42.62%), 178.0859 (100%), 123.0036 (45.10%) (, calcd. for C_4H_8ClS, 123.0035).

1-(p-Nitrophenyl)-3-{S-(2-chloroethyl)thioethyl} triazene 59

(1.1 g, 38%), m.p. 50-52°, ν_{max} 3173.5, 3135.8 and 1595. δ ($CDCl_3$, -15°) 2.95 (m, 4H, CH_2-S-CH_2), 3.7 (t, 2H, CH_2-Cl), 4.0 (t, 2H, N-CH_2), 7.19 - 8.4 (m, 4H, aromatic), 8.6 and 9.6 (br, 2S, 1H, exchangeable, N-H), M^+ 288.0444 (calcd. for $C_{10}H_{13}ClN_4O_2S$, 288.0448), 260.0389 (13.39%) (M-N_2, calcd. for $C_{10}H_{13}ClN_2O_2S$, 260.0392), 224.0619 (3.45%), ((M-N_2)-HCl calcd. for $C_{10}H_{12}N_2O_2S$, 224.0622), 138.0429 (100%); 123.0034 (31.60%) (, calcd. for C_4H_8ClS, 123.0035).

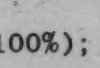
S-(3-hydroxypropyl)thioethylamine 60

To a solution of 3-mercaptopropanol (3 g, 32 mmol) in 25 ml of 95% ethanol was added 4.5 g of potassium hydroxide dissolved in a minimum amount of water and the mixture was heated to 90°C under stirring. To the stirred hot solution was added dropwise a saturated solution of 2-chloroethyl-aminehydrochloride (4.0 g, 34 mmol) in 95% ethanol. The reaction mixture was stirred at 90°C for 2 h. The stirring was continued overnight at room temperature. The solid was collected and the filtrate was concentrated under reduced pressure on a rotary evaporator. The product distilled (152°-153°, 0.2 mm) as a colorless liquid (yield 58%). δ($CDCl_3$) 1.8 (m, 2H), 2.5 - 3.0 {m, 9H, (3H exchangeable)}, 3.66 (t, 2H, CH_2-O).

S-(3-chloropropyl)thioethyamine 61

Amino alcohol 60 (2 g), was dissolved in $CHCl_3$ (20 ml) and saturated with HCl gas at 0°. Thionylchloride (10 ml) diluted with $CHCl_3$ (10 ml) was added to the amine hydrochloride at 0° and the reaction mixture was stirred at room temperature for 48 h. On evaporating the solvent and excess of thionylchloride the tarry residue solidified when triturated with anhydrous ether. The solid was recrystallized from chloroform/ether.

1-(p-Cyanophenyl)-3-{S-(3-chloropropyl)thioethyl} triazene 62

Triazene 62 was prepared following the general procedure. (0.9 g, 32%), m.p. 60-61°; ν_{max} 3178.6, 3156.4, 2223.8 and 1605 cm^{-1}; δ (CD_2Cl_2, -25°) 2.05 (q, 2H, CH_2-CH_2-CH_2), 2.6 - 2.95 (m, 4H, CH_2-S-CH_2); 3.7 (t, 2H, CH_2-Cl); 3.95 (t, 2H, N-CH_2); 7.15 - 7.75 (m, 4H, aromatic); 8.7 and 9.6 (br, 2s, exchangeable, N-H); M^+ 282.0711 (2.80%) (calcd. for $C_{12}H_{15}ClN_4S$, 282.0706); 254.0648 (13.66%) ($M-N_2$, calcd. for $C_{12}H_{15}ClN_2S$ 254.0645); 118.0531 (100%); 137.0182 (29.58%) ( , calcd. for $C_5H_{10}ClS$, 137.0183).

1-(p-Cyanophenyl)-3-{O-(2-chloroethyl)ethoxy}triazene 63

(1.6 g, 63%), m.p. 60°, ν_{max} 3179.2, 3158.3, 2220.3, 1609 cm^{-1}. (δ, $CDCl_3$, -50°), 3.6 - 4.1 (m, 8H, N-CH_2-CH_2-OCH_2CH_2Cl), 7.15 - 7.80 (m, 4H, aromatic), 8.85 and 10.10 (t and s respectively, 1H, exchangeable, N-H); M^+ 252.0772 (10.67%) (calcd. for $C_{11}H_{13}ClN_4O$, 252.0777), 226.0688 (1.21%) (M-N_2, calcd. for $C_{11}H_{13}ClN_2O$, 226.0687), 118.0529 (79.05%), 102.0343 (100%), 107.0262 (8.48%) (, calcd. for C_4H_8ClO, 107.0264); anal. calcd. for $C_{11}H_{13}ClN_4O$: C = 52.27%; H = 5.14%, N = 22.17, O = 6.33 Found C = 52.35%; H = 4.90% , N = 22.60%, O = 6.70.

1-(p-Cyanophenyl)-3-{S-(2-hydroxyethyl)thioethyltriazene 64

The compound was prepared following the general procedure. The diazonium salt solution obtained from the diazotization of 10 mmol of p-aminobenzonitrile was allowed to react with 9 mmol of S-(2-hydroxyethyl)thioethylamine after neutralizing the excess acid with $CaCO_3$. The product was extracted with CH_2Cl_2, the combined extracts dried ($MgSO_4$) and the solvent was removed under vacuum. The gummy residue was dissolved in CH_2Cl_2 and cooled to -30°. On adding petroleum ether a solid precipitated which reverted to a gummy liquid during filtration (yield 70%). ν_{max} 3410 (OH, br), 3180, 3160, 2223 and 1608 cm^{-1} δ (CD_2Cl_2, -30°) 2.8 (m, 4H, CH_2-S-CH_2), {3.72 (t, CH_2-OH), 3.9 (t, -N-CH_2-) Total integration 5H, includes -OH} 7.1 - 7.85 (m, 4H, aromatic), 9.45 and 11.7 (t and s(br) respectively, 1H, NH).

δ (CD_2Cl_2 32°), 2.8 (m, 5H, CH_2-S-CH_2 + OH), 3.72 and 3.9 (2t, 4H, CH_2-OH and N-CH_2 respectively), 7.1 - 7.85 (m, 4H, aromatic), 9.0 (s, br, 1H, N-H). On adding D_2O the multiplets at 2.8 and 3.8 (two triplets at 3.72 and 3.9 together) each integrated for 4 H. The broad singlet at 9.0 ppm disappeared. M^+/e 232.0756 (M-H_2O, calcd. for $C_{11}H_{12}N_4O$, 232.0783).

S-(2-hydroxyethyl)-1,1-dideuteriothioethylamine 65

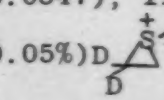
This amino alcohol was prepared by condensing 2-chloro-1,1-dideuterioethylaminehydrochloride 29 with 2-mercaptoethanol following the procedure reported for the corresponding protium compound.[102]

S-(2-chloroethyl)-1,1-dideuteriothioethylamine Hydrochloride 66

Amino alcohol 65 was chlorinated following the literature procedure.[102]

1-(p-Cyanophenyl)-3-{S-(2-chloroethyl)-1,1-dideuteriothioethyl}triazene 67

Triazene 67 was prepared following the procedure for the corresponding protium analog (yield 55%), m.p. 70-71° ν_{max} 3180.9, 3157.3, 2223.1 and 1605 cm^{-1}; (δ, CD_2Cl_2, -20°), 2.80 - 3.05 (m, 4H, CH_2-S-CH_2); 3.6 - 3.85 (m, 2H, CH_2-Cl); 7.15 - 7.80 (m, 4H, aromatic); 8.65 and 9.55 (br, 2s, exchangeable, N-H); δ ($CHCl_3$, $^2H_1$) 3.92 (s, N-CD_2);

M^+ 270.0675 (3.29%) (calcd. for $C_{11}H_{11}D_2N_4ClS$, 270.0675); 242.0615 (4.45%) ($M-N_2$, calcd. for $C_{11}H_{11}D_2ClN_2S$, 242.0613); 206.0851 (1.19%) ($(M-N_2)-HCl$, calcd. for $C_{11}H_{10}D_2N_2S$, 206.0847); 118.0534 83.62%); 102.0350 (98.28%); 125.0155 (40.05%)  calcd. for $C_4H_6D_2ClS$, 125.0161).

Alkylation of Triacetyluridine by Triazene 56

Triacetyluridine (.095 g, 0.25 mmol) and triazene 56 (0.134 g, 0.5 mmol) were dissolved in anhydrous 1,2-dimethoxyethane (5 ml) in an air-tight container. The reaction mixture was stirred at 50° for 48 h. The solvent was removed under vacuum and the residue was subjected to chromatography on silica using a solution of acetone and benzene (1:5) as eluant affording the N^3-alkylated product 83. (yield, 5%), ν_{max} 1751.4, 1714.1 and 1675.8 cm^{-1}. δ ($CDCl_3$, 400 MHz), 2.15 (m, 9H, 3-CH_3), 2.8 and 2.95 (2t, 4H, CH_2-S-CH_2), 3.7 (t, 2H, Cl-CH_2), 4.15 (t, 2H, CH_2-N-), 4.35 (s, 3H), 5.35 (m, 2H), 6.82 (d, 1H), 6.0 (d, 1H), 3.78 (d, 1H); m/e 456·1204 (M-HCl, calcd. for $C_{19}H_{24}N_2O_9S$, 456.1202); CIMS (NH_3) 518 (M + 18); uv (CH_3OH) max. 273 nm.

N-3 Alkylated Uridine From Triazene 67, 83'

ν_{max}, 1751.4, 1714.1 and 1675.8 cm^{-1}. δ ($CDCl_3$, 400 MHz), 2.15 (m, 9H, 3-CH_3), 2.8 (s, 2/3 H, -S-$\underline{CH_2}$-CD_2NH_2), 2.95 (t, S-$\underline{CH_2}$-CH_2Cl), 3.7 (t, 2H, CH_2Cl), 4.15 (s, 1/3 H, -N-CH_2-CD_2-S), 4.35 (s, 3H), 5.35 (m, 2H), 6.82 (d, 1H),

6.0 (d, 1H), 7.38 (d, 1H), CIMS (NH_3) 512 (M + 18); uv (CH_3OH) max. 273 nm.

Esterification of 3,5-dinitrobenzoic Acid by Triazene 56

To the stirred suspension of triazene 56 (0.6 g, 2 mmol) in anhydrous ether (10 ml) was slowly added 3,5-dinitrobenzoic acid (0.6 g, 3 mmol) dissolved in anhydrous ether (20 ml). The stirring was continued for 10 hours. The reaction mixture was filtered and the solvent was removed under reduced pressure. The viscous residue was subjected to chromatography on florisil using benzene as eluant. This procedure afforded the following esters.

S-(2-chloroethyl)thioethyl-3,5-dinitrobenzoate

(0.5 g, 68%), m.p. 60-61°; ν_{max} (CH_2Cl_2) 1736.4, 1550 and 1345 cm^{-1}. δ ($CDCl_3$) 3.05 (m, 4H, CH_2-S-CH_2), 3.7 (t, 2H, CH_2-Cl), 4.54 (t, 2H, O-CH_2), 9.10 - 9.25 (m, 3H, aromatic); m/e 332.9935 (0.55%) (M-1, calcd. for $C_{11}H_{10}ClN_2O_6S$, 332.9948).

Anal. calcd. for $C_{11}H_{11}ClN_2O_6S$, C = 39.52%, H = 3.29, N = 8.38

Found c = 39.52%, H = 3.31%, N = 8.17%

S-(2-chloroethyl)1',1'- and 2',2'-dideuteriothioethyl-3,5-dinitrobenzoates 84 + 85

m.p. 60°, ν_{max} (CH_2Cl_2) 1736.1, 1550 and 1345 cm^{-1}. δ ($CDCl_3$), 3.02 (m, 2.44 H, CH_2-S-CH_2 + CD_2-S-CH_2),

3.7 (t, 2H, CH_2-Cl), 4.65 (s, 1.56 H, O-CH_2), 9.15 - 9.3 (m, 3H, aromatic); δ ($CHCl_3$, $^2H_1$) 4.65 (s, 1.22 $^2H_1$, O-CD_2), 3.02 (s, .88 $^2H_1$, CD_2-S-); M^+ 336.0148 (0.54%) (calcd. for $C_{11}H_9D_2N_2O_6S$, 336.0152).

General Procedure for the Esterification of Diethyl Phosphate by a Triazene

Diethyl phosphate (2 mmol) dissolved in ether (10 ml) was added dropwise to a stirred suspension of the triazene (2 mmol) in ether (10 ml) at -20°. After the addition was completed the reaction mixture was allowed to warm up to 0°C and the stirring was continued for 30 min. The solvent was removed and the residue was purified by chromatography on florisil using a mixture of benzene and acetone as eluant. Initially 5% acetone in benzene eluted p-aminobenzonitrile. The proportion of acetone in the eluant was increased gradually during the chromatography. This procedure afforded the following phosphate triesters.

Diethyl, S-(2-chloroethyl)thioethyl phosphate 86 from triazene 56

A purple viscous liquid, yield 8%. δ ($CDCl_3$), 1.35 (t, 6H, 2 Me), 2.85 (m, 4H, CH_2-S-CH_2), 3.62 (t, 2H, CH_2-Cl), 4.1 (m, 6H, 3 O-CH_2), m/e 241.0617 (1.33%), (M-Cl, calcd. for $C_8H_{18}O_4PS$, 241.0663), 240.0578 (9.07%) (M-HCl, calcd. for $C_8H_{17}O_4PS$, 240.0585), ClMS (NH_3) 294 (M + 18).

Diethyl, S-(3-chloropropyl)thioethyl phosphate 87

from triazene 62

Yield 6%, (δ, $CDCl_3$), 1.35 (t, 6H, 2 Me), 2.05 (m, 2H, CH_2), 2.75 (m, 4H, CH_2-S-CH_2), 3.65 (t, 2H, CH_2-Cl), 4.15 (m, 6H, 3 O-CH_2). CIMS (NH_3) 308 (M + 18).

Diethyl, O-(2-chloroethyl)ethoxy phosphate 88

from triazene 63

Yield 65%, ν_{max} (film) 1270 cm^{-1} (P = O), 1020 cm^{-1} (P-O) δ ($CDCl_3$), 1.32 (t, 6H, CH_3), 3.5 - 4.3 (m, 12H, CH_2). Anal. calcd. for $C_8H_{19}PO_5Cl$ (MW + proton 261.0659, MW - proton 259.0502); C = 36.92; H = 6.92. Found (261.0653, 259.0498, mass spectrum); C = 36.81, H = 6.77. CIMS (ammonia) 278 (M + 18).

Alkylation of p-aminobenzonitrile

I. A mixture of p-aminobenzonitrile (10 mmol) and bis(2-chloroethyl)sulfide (10 mmol) in 2 ml of aqueous ethanol was placed in a reactivial. After vigorous shaking a turbid solution was obtained. The reaction mixture was maintained at 37° for 72 h. On cooling the reaction mixture needle-shaped crystals precipitated. The solid product was collected and identified spectroscopically as 82. The filtrate was evaporated to dryness and the residue was separated by chromatography on silica using benzene/5% acetone as eluant. One of the products was identified as 72.

II. A mixture of p-aminobenzonitrile (10 mmol) and bis(2-chloroethyl)sulfide (10 mmol) in 2 ml of anhydrous 1,2-dimethoxyethane was placed in a reactivial. After shaking a clear solution was obtained. The reaction mixture was maintained at 37° for 96 h. On cooling the reaction mixture the compound 82 precipitated. The product was collected, and the filtrate was evaporated to dryness. The residue was purified by chromatography as in the case of I described above affording, bis-{2-(p-cyanoanilino)-ethyl} sulfide 82.

ν_{max} 3360, 2220 and 1610 cm^{-1}

δ ($CDCl_3$) 2.8 (t, 4H, CH_2-S-CH_2), 3.34 (m, 4H, N-CH_2), 6.48 (t, 2H, exchangeable, N-H), 6.6 - 7.44 (m, 8H, aromatic). ClMS (NH_3) 340 (M + 18).

Reaction of N^3-alkyltriacetyluridine 83 and phosphotriesters 86, 87 and 88 with γ-(p-nitrobenzyl)pyridine (NBP)

10% Stock solutions of 83, 86, 87 and 88 were prepared in acetone. The reactions were carried out in 10 ml screw capped test tubes containing water (2 ml), cacodylate buffer pH 7.0 (1 ml), NBP (400 µℓ, 5% in acetone) and 10 µℓ of stock solution. The reaction mixtures were immersed in a hot water bath for 20'. Subsequently the reaction mixtures were cooled and 0.25 N KOH solution (1.5 ml) was introduced in to each test tube.

METHODS

Studies Related to the Aqueous Decomposition of S-(2-chloroethyl)thioethyaryltriazenes

Identification of Volatile Products

GC/MS analyses were performed on an AEI MS-12 spectrometer using helium gas flow rate of 20 ml/min. Samples were injected onto a 4 ft 10% carbowax 20 M 80-100 WAW-DMCS 5830 column. The column was heated at 70° for 5 min and was heated further with a rate of 4°/min up to 150°; this temperature was maintained until all volatile products had been swept from the column. Two volatile products 2-chloroethylvinylsulfide 73 and bis(2-chloroethyl)sulfide 68 were identified using this procedure. The observed retention times for 73 and 68 were 11.37 and 25.6 min respectively.

Identification of Bis(2-hydroxyethyl)sulfide 69 in the Aqueous Decomposition of 56

GC analysis was performed on a Hewlett-Packard Model 5840 A gas chromatograph equipped with flame ionization detector. An aqueous solution of the decomposition products was injected onto a 6' column of Porapak Q (80-100 MESH) maintained isothermally at 240°C with helium gas flow rate of 19 ml/min. The observed retention time for 69 was 26.23 min.

A sample of triazene (50 mmol) suspended in 0.1 M phosphate buffer (1 ml pH 7.2) in 5 ml capacity screw capped reactivials was allowed to decompose for 72 h. The vial was cooled in ice and 0.5 ml of dichloromethane was injected into the vial and shaken thoroughly. The dichloromethane solution (2 µℓ) was injected for GC and GC/MS analyses. For the identification of 69 2 µℓ of the aqueous solution was analyzed.

CHAPTER V.

DNA INTERSTRAND CROSS-LINKING BY S-(2-CHLOROETHYL)THIOETHYLTRIAZENES

The application of the ethidium fluorescence assay has been extended to detect and estimate the amount of covalently linked complementary DNA (CLC-DNA) produced after reaction with an appropriate compound.[103,104] Using the assay, aliquots of cross-linked DNA are analyzed for CLC sequences by diluting them in a solution of ethidium bromide buffered to pH 11.8. The fluorescence of the DNA-ethidium bromide solution is measured to obtain an estimate of the total DNA concentration. The solution is then heat denatured (96°/3 min), cooled quickly (0°C) then equilibrated to 22° and the fluorescence of the solution is again measured. Under these conditions separated DNA strands do not reanneal. CLC-sequences can reanneal since the cross-link may serve as a nucleation point. Reannealing results in the formation of double stranded DNA which binds ethidium bromide. The ratio of the fluorescence after the heat denaturation and cooling cycle to the fluorescence before heat denaturation is then a measure of the extent of covalent interstrand cross-linking. The assay is conducted at pH 11.8 to prevent spontaneous formation of short intrastrand bihelical structures after heating and cooling of separated single strands of DNA. Such structures are unstable at pH 11.8 when compared with those formed by

CCC-DNA and are due to a certain amount of self-complementarity within strands of naturally occurring DNA's.[40,105] The validity of this assay procedure for detecting the formation of CLC-DNA as a result of a chemical cross-linking event has been confirmed by experiments with the enzyme S_1-endonuclease.[103] This enzyme specifically cleaves single-stranded DNA and is essentially inactive on duplex DNA. Therefore, it distinguishes DNA which is renaturable because of a chemical cross-link from DNA which separates into single-strands upon heating. Cross-linking without strand scission is depicted in Figure 11.

The assay for alkylation is complicated when the alkylating agent also cross-links DNA. If the alkylating agent cross-links the DNA, the loss of fluorescence that normally occurs after heat denaturation of the alkylated DNA is not observed instead a return of fluorescence is observed as shown for example in Figure 11.

S-(2-chloroethyl)thioethyltriazenes 56, 57, 58 and 59 covalently cross-link DNA under physiological conditions. Although the same reactions occurred more efficiently at 37°C, in a selected case a significant amount of cross-linking was observed at 0°C. The rate of cross-linking increases with decreasing pH in the range 5-10 (Figure 12) which is parallel to their pH dependent rate of decomposition measured polarographically and is in accord with the suggested mechanism of reaction for this class of triazenes (see Chapter IV). The cross-links were observed to be

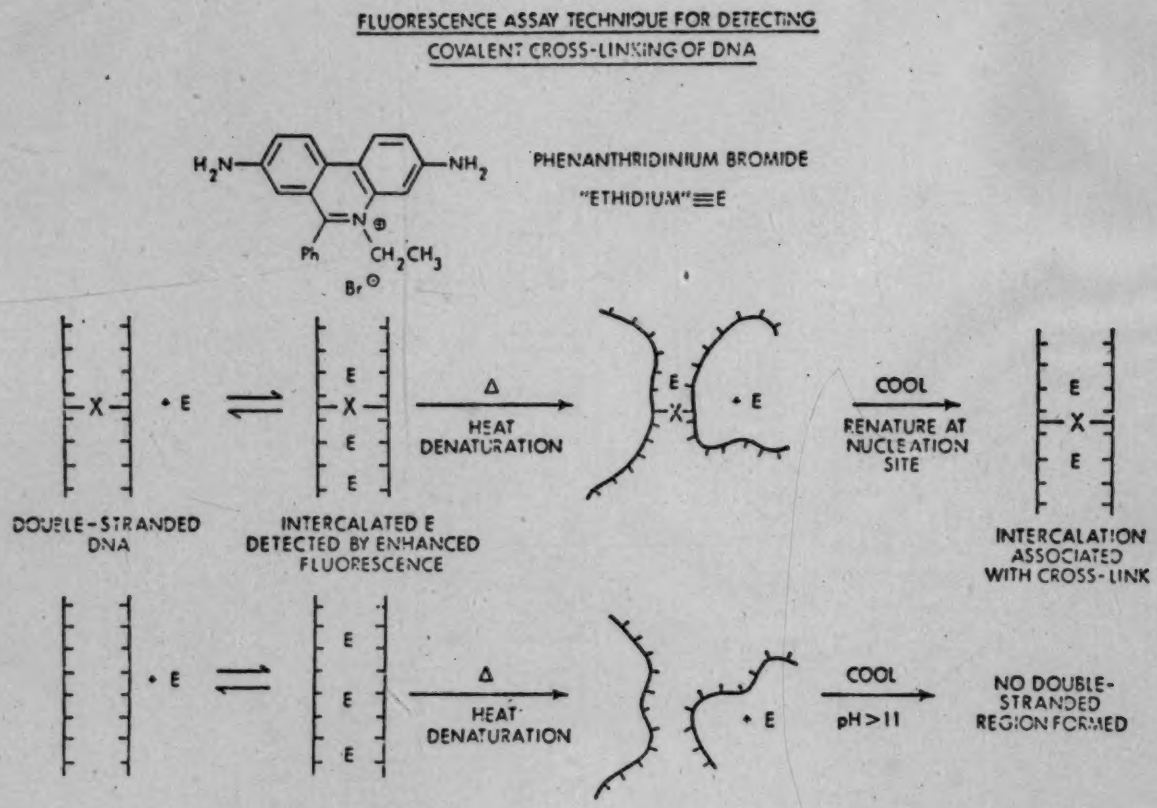


Figure 11. Ethidium bromide assay for the detection of DNA interstrand cross-linking of λ-DNA.

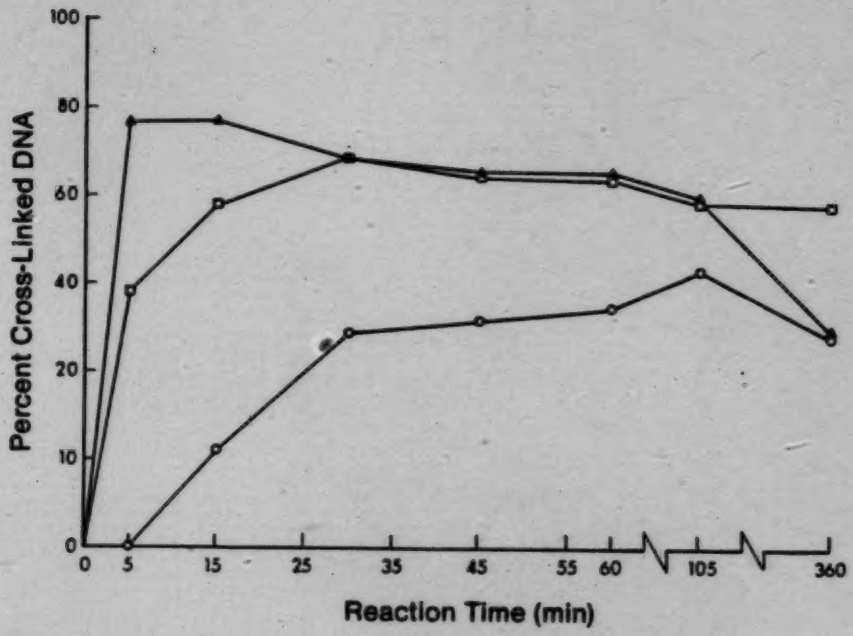


Figure 12. pH Dependence of DNA cross-linking by S-(2-chloroethyl)thioethytriazene 56. Reactions were performed at 37°C in a total volume of 200 µℓ buffered at pH 4.7 (▲-▲), pH 7.2 (□-□), pH 10.1 (O-O) with potassium phosphate and contained 1.0 A_{260} of λ DNA and 5 mM triazene.

stable for over 48 h in 0.15 M NaCl and 0.015 M sodium citrate, conditions which have been reported[106,107] to reverse the interstrand cross-links produced by carzinophillin. This suggests the formation of two covalent bonds. Unlike the observations with 2-chloroethylnitrosoureas[45] the two alkylating steps in the process of interstrand cross-linking are kinetically less distinguishable by ethidium fluorescence assay. However, during the alkylation of PM2-CCC-DNA (discussed at length) some indication of a 2 step reaction in the interstrand cross-linking was obtained.

Since one of the two alkylating steps in the process of interstrand cross-linking is due to the sulfur half-mustard portion of the alkylated component of DNA it is conceivable that the initial site of alkylation to complete the event is not crucial, as demonstrated by model studies in Chapter IV. The uridine derivative 83 and the phosphate triester 86 representing an alkylated base and an alkylated phosphate diester of a DNA strand, respectively, alkylate DNA at comparable rates under physiological conditions.

Although a time dependent rate of alkylation of supercoiled PM2-CCC-DNA by triazene 56 could not be obtained owing to a progressive interstrand cross-linking, a substantial decrease of fluorescence after heat denaturation (66%) during the first 5 min of incubation of the reaction mixture indicated that the primary alkylation may have somewhat lower activation energy than the second step, and that the secondary alkylation is a slightly delayed process. When

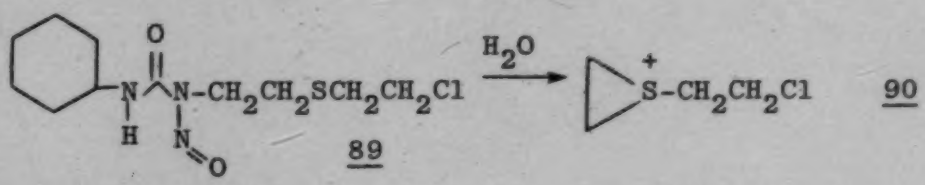
the supercoiled PM2-CCC-DNA was relaxed with calf thymus topoisomerase, the two steps became kinetically indistinguishable. The alkylation of PM2-CCC-DNA with respect to its topology has not been studied and any comments on the observed dependency of the rate of cross-linking on the topology of DNA is beyond the scope of this dissertation.

The triazenes 62, 63 and 64 gave no indication of cross-linking although a time dependent progressive alkylation of DNA was observed. The observations are in accord with the model studies conducted in Chapter IV.

DNA Single Strand Breaks Induced by S-(2-Chloroethyl)-thioethyltriazenes

A progressive production of base labile sites was observed when PM2-CCC-DNA relaxed with calf thymus topoisomerase was incubated at pH 7, 37° with triazene 56 and assayed at pH 11.8. What appeared to be mainly Type I SSS was detectable only after 60 min of incubation and gradually increased over the next 60 min. Incubation of the assay solution at 37° for a period of up to 3 hrs did not show any enhancement in the extent of SSS indicating a complete absence of Type II SSS. Addition of endonuclease VI to the reaction mixture followed by assay at pH 8 buffer independently confirmed that the triazene 56 did not cause any Type II SSS. These results show that the sulfur mustard triazenes differ in behavior from the 2-haloalkyltriazenes which exhibit only Type II SSS.

The aqueous decomposition of triazenes and the corresponding nitrosoureas has indicated consistently that some of the alkylating species generated from the two classes of compounds are either the same or chemically equivalent. The aqueous decomposition of nitrosourea 89 has confirmed the generation of episulfonium species 90.[108] This provided an opportunity to compare the action of the



89 90

nitrosourea 89 on DNA with that of structurally related triazene 56.

When nitrosourea 89 (known to produce cross-links)[45] was incubated with relaxed PM2-CCC-DNA and assayed at pH 11.8 buffer both types of single strand scissions were observed (Figure 13). The depurination caused by nitrosourea 89 was confirmed by employing endonuclease VI. Therefore despite the fact that the triazene 56 and nitrosourea 89 both generate episulfonium species 90 in their aqueous decompositions, the preferred sites of alkylation by two types of compounds in the presence of DNA appear to be significantly different. It is also possible that owing to the greater reactivity of 2-chloroethyltriazene 16 compared with that of S-(2-chloroethyl)thioethyltriazene 56 the former shows less selectivity in terms of site-preference while alkylating DNA. The alkylating species from 16 may react with

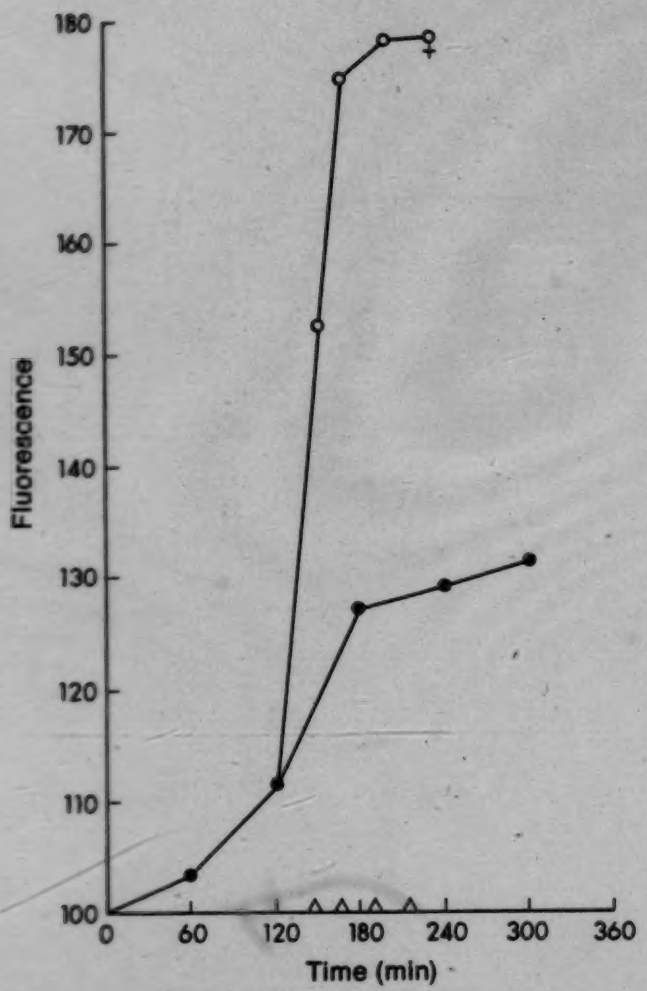
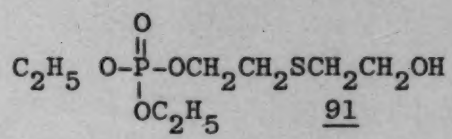


Figure 13. Reaction of PM2-CCC-DNA 1.0 A_{260} in 0.05 M cacodylate buffer pH 7.0 at 37°C with 5 mM nitrosourea 89. Measurement of Type 1 SSS (●), followed after 120 minutes of reaction with; (O) endonuclease VI; (+) 90 minutes incubation at 37° pH 11.8; (Δ) control (relaxed PM2-CCC-DNA with endonuclease VI).

sites that lead to depurination and depyrimidination (see Chapter III). S-(2-chloroethyl)thioethyltriazenes in general due to their greater stability compared with those of the corresponding 2-chloroethyltriazenes show higher selectivity for the primary sites that can predominantly be the phosphate residue of DNA. In contrast, the selective base promoted decomposition of nitrosoureas makes the purine and pyrimidine bases as its preferred site for alkylation, a property that has been further substantiated by the observed depurination of DNA due to the nitrosourea 89.

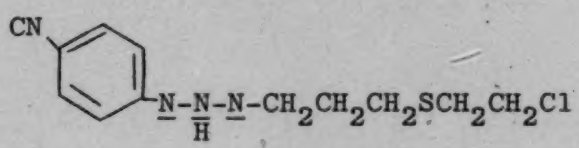
The secondary sites of alkylation to complete the cross-linking in the case of representative triazene 56 may include the purine and pyrimidine bases. However, the sites such as N-3 position of deoxyadenosine and N-7 position of deoxyguanosine that lead to facile depurinations appear to have been precluded even as secondary sites of alkylation.

The origin of Type I SSS observed with triazene 56 is not clear. However, it may be noted that S-(2-hydroxyethyl)thioethyltriazene 63 causes an extensive Type I SSS in a manner observed with 2-hydroxyethyltriazenes discussed in Chapter III. Whether a slow formation of S-(2-hydroxyethyl)thioethylphosphotriester 91 which like 2-hydroxyethylphosphotriester[77,78] provides an intramolecular assistance in the hydrolysis of phosphate residue of DNA leading to Type I SSS can not be said with certainty.



91

It was desirable to know the significance of the distance between the two sites of alkylation to complete the process of interstrand cross-linking in terms of kinetics. Also, it was decided to investigate if there was any assistance from sulfur in the generation of initial alkylating species. An appropriate combination of triazenes for this purpose would be S-(3-chloropropyl)thioethyltriazene 62 and S-(2-chloroethyl)thiopropyltriazene 92 along with



92

S-(2-chloroethyl)thioethyltriazene 56. However, the triazene 92 turned out to be unstable and these studies were carried out on the corresponding nitrosoureas 93 96 and 89.

Decomposition of Nitrosoureas 95 and 96

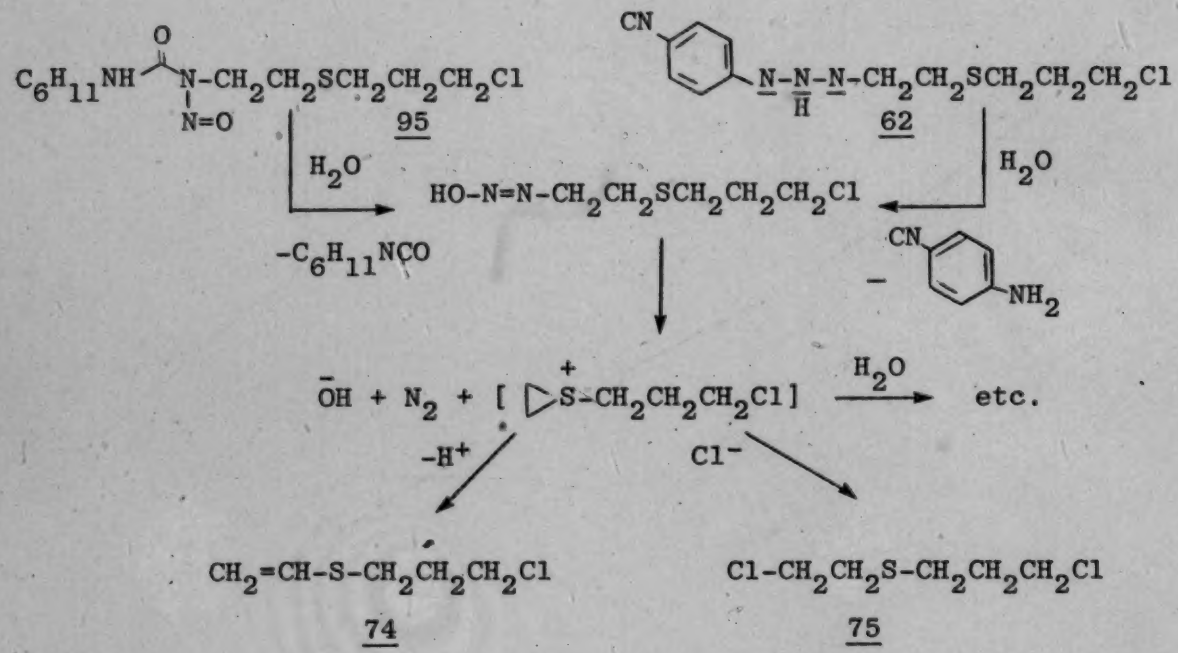
The decompositions were carried out at pH 7.2, 37°C. One milliliter of a 50 mmol nitrosourea was allowed to decompose in a sealed vial for 72 hrs and the products were analyzed by GC/MS following the procedure used for the structurally related triazenes described in the experimental section of Chapter III.

Nitrosourea 95 gave 3-chloropropyl vinylsulfide 74 and 2-chloroethyl,3-chloropropylsulfide 75, accounting for 73% and 8% of the alkyl side-chain, respectively. Cyclohexyl isocyanate accounted for most of the nitrosourea (Scheme 23). Nitrosourea 96 gave 2-chloroethyl,2-propylene-sulfide 97 accounting for 69% of the alkyl side-chain. The isomeric product 3-chloropropyl,vinyl sulfide 74 and 2-chloroethyl,3-chloropropyl sulfide 75 accounted for 2% and 15% of the side-chain, respectively. Cyclohexyl isocyanate accounted for over 90% of the nitrosourea (Scheme 24).

The polarographic rates of decomposition of nitrosoureas 95 and 96 determined under identical conditions are very close (Table 13). This observation indicates that the initial decomposition of these compounds proceeds via a labile intermediate such as a diazohydroxide formed in a rate determining step (Schemes 23 and 24).

The nitrosourea 95, like the corresponding triazene 62, failed to form any interstrand cross-links as anticipated and for the reasons discussed earlier. When the nitrosoureas 89 and 96 were allowed to react with DNA under physiological conditions (pH 7.2, 37°) the rate of interstrand cross-linking produced by the former was higher compared with the latter as observed over a period of 20 min (Figure 14). This indicates that perhaps there is an optimum chain length of a bifunctional alkylating species for an efficient cross-linking of the complementary strands of DNA. However, further investigation is necessary for a

SCHEME 23


C6H11NH
N-CH2CH2SCH2CH2CH2Cl
N=O
95
CN
N-N-N-CH2CH2SCH2CH2CH2Cl
H
62
H2O
HO-N=N-CH2CH2SCH2CH2CH2Cl
-C6H11NCO
CN
NH2
OH + N2 + [S-CH2CH2CH2Cl]
H2O
etc.
-H+
Cl-
CH2=CH-S-CH2CH2CH2Cl
74
Cl-CH2CH2S-CH2CH2CH2Cl
75

SCHEME 24

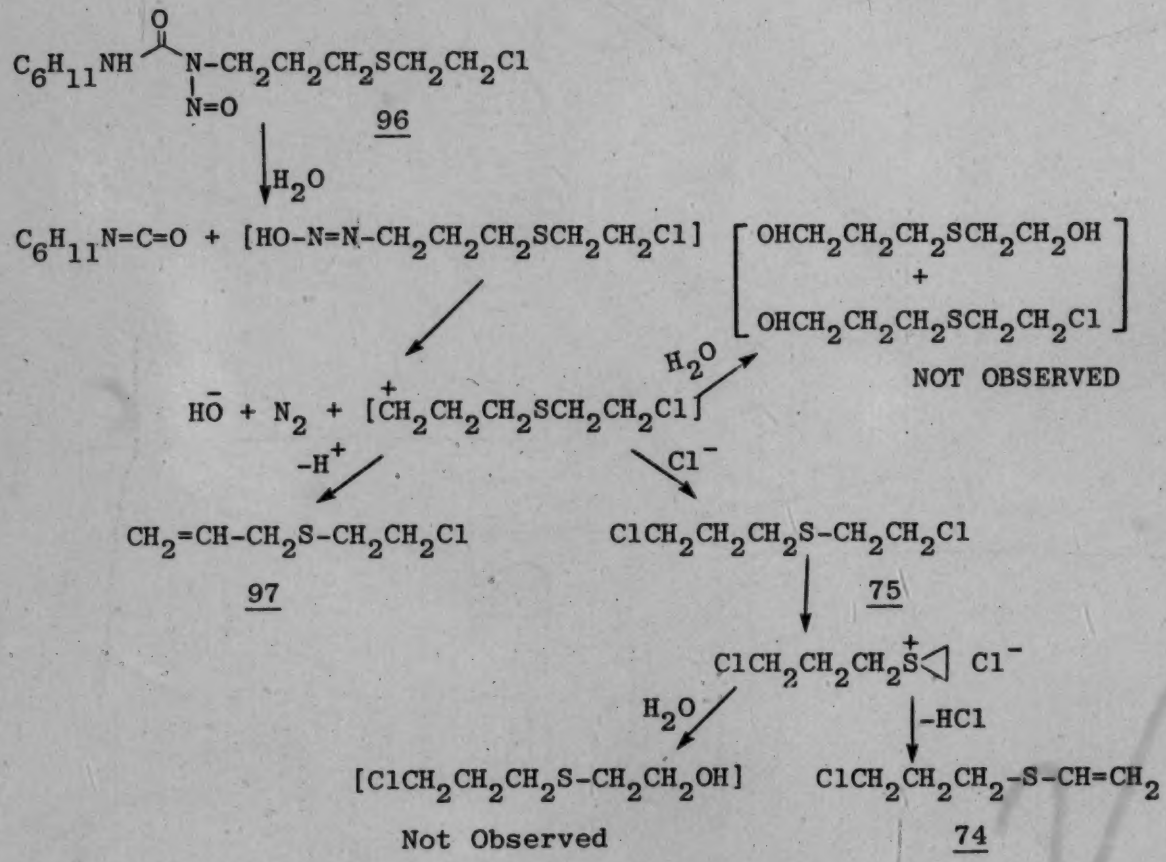

C6H11NH
O
N-CH2CH2CH2SCH2CH2Cl
N=O
96
H2O
C6H11N=C=O + [HO-N=N-CH2CH2CH2SCH2CH2Cl]
[OHCH2CH2CH2SCH2CH2OH + OHCH2CH2CH2SCH2CH2Cl]
NOT OBSERVED
H2O
HO⁻ + N2 + [+CH2CH2CH2SCH2CH2Cl]
-H+
Cl⁻
CH2=CH-CH2S-CH2CH2Cl
97
ClCH2CH2CH2S-CH2CH2Cl
75
ClCH2CH2CH2S+ Cl⁻
H2O
-HCl
[ClCH2CH2CH2S-CH2CH2OH]
Not Observed
ClCH2CH2CH2-S-CH=CH2
74

TABLE 13

POLAROGRAPHIC BEHAVIOR OF NITROSOUREAS 95 AND 96 DETERMINED IN 5% AQUEOUS ACETONITRILE

	$E_{\frac{1}{2}}$ [1]	$E_{\frac{1}{2}}$ [2]	$t_{\frac{1}{2}}$ (sec.)
Nitrosourea #95	-0.952	-1.444	1233.6
Nitrosourea #96	-0.960	-1.350	1157.4

[1] D.C.

[2] D.P.

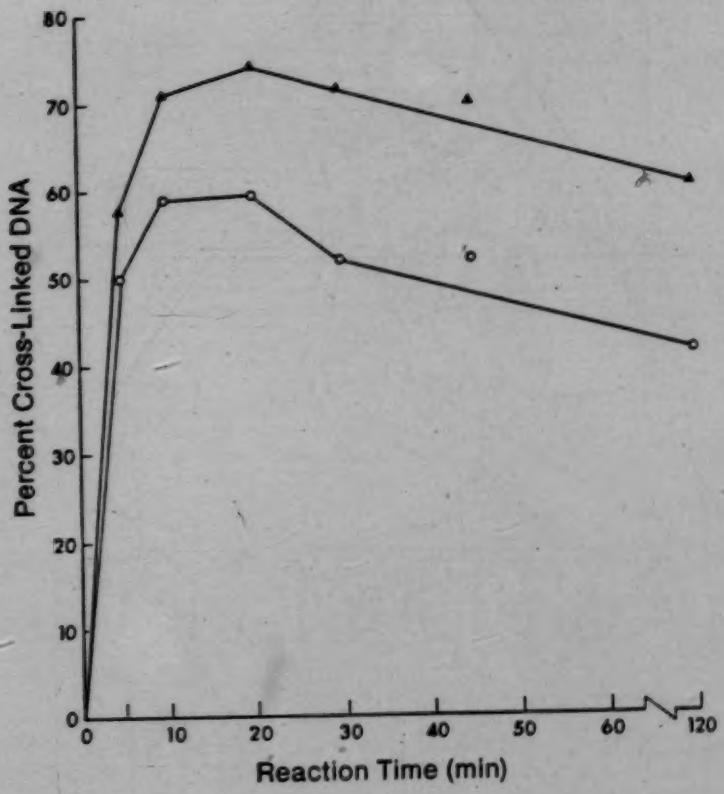


Figure 14. DNA interstrand cross-linking of λ DNA 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 37°C with 5 mM (▲) nitrosourea 89 and (O) nitrosourea 95.

conclusive statement in this regard.

The triazenes 56 and 57 exhibit significant anti-leukemic properties in animal test systems. Structural modifications in the side chains of the triazene 56 and the nitrosourea 89 invariably resulted in the loss of activity (Table 14).

Consideration of DNA Interstrand Cross-Linking by 3-(2-Haloethyl)Aryltriazenes

By analogy with 2-chloroethylnitrosoureas[45] one might anticipate the detection of DNA interstrand cross-links by the 3-(2-chloroethyl)aryltriazene 16. Although, as has been pointed out in chapter III, extensive alkylation of both the bases and the phosphate residues of DNA by the triazenes is apparent the interstrand cross-links were not detectable. A number of factors seem to be involved here. First under comparable conditions the triazenes decompose much more readily and thus give rise to a higher local concentration of electrophiles than the comparable nitrosoureas e.g. $t_{\frac{1}{2}}$ for triazene 16 is 104 sec compared with 474 sec for bis(2-chloroethyl)nitrosourea (BCNU).[68] Second it is the 2-chloroethylation of the DNA bases by 2-haloethylnitrosourea which result in cross-links.[45,109] However the triazenes, owing to their acidic activation,[75] show a preferential reactivity towards the phosphate residues of nucleic acids. This is reflected in the more extensive degradation of RNA by triazene 16 (50% in 1 h) compared with

TABLE 14

#	Compound	Activity Against P388 Leukemia[a] % T/C
56	1-(p-Cyanophenyl)-3-{S-(2-chloroethyl)thioethyl}triazene	170
57	1-(p-Acetylphenyl)-3-{S-(2-chloroethyl)thioethyl}triazene	179
63	1-(p-Cyanophenyl)-3-{S-(2-chloroethyl)ethoxy}triazene	107
64	1-(p-Cyanophenyl)-3-{S-(2-hydroxyethyl)thioethyl}triazene	138
89	1-{2-[(2-Chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea	224*
95	1-{3-[(2-Chloroethyl)thio]propyl}-3-cyclohexyl-1-nitrosourea	118*
96	1-{2-[(3-Chloropropyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea	102*

*Compounds were tested against leukemia L1210.

[a]Assays for activity against lymphoid leukemia P388 and leukemia L1210 performed according to specifications established by the Cancer Chemotherapy National Service Centre.[111]

that produced by BCNU (16% in 1 h). Third the onset of interstrand cross-linking by 2-chloroethylnitrosoureas can normally be observed after about 2 hrs of incubation at 37°.[45] During this time period the extensive amounts of alkylation of the phosphate residues of the nucleic acids (indicated by both the RNA and DNA experiments) leading to strand scission may prevent the detection of any interstrand links that might be formed by the aryltriazenes.

EXPERIMENTAL

1-{2-[(2-chloroethyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea 89 was prepared following the literature procedure.[45]

S-(2-hydroxyethyl)thiopropylamine 93

2-Mercaptoethanol was allowed to react with 3-chloropropylamine following the procedure described for the preparation of S-(3-hydroxypropyl)thioethylamine 60. The amino alcohol distilled as a clear liquid at 125°(0.2 mm).

S-(2-chloroethyl)thiopropylamine Hydrochloride 94

The amino alcohol 93 was chlorinated following the procedure described for the chlorination of S-(2-hydroxyethyl)thioethylamine.[102]

1-{3-[(2-Chloroethyl)thio]propyl}-3-cyclohexyl-1-nitrosourea 95

S-(2-chloroethyl)thiopropylamine hydrochloride 94 (250 mg, 1.3 mmol) was deprotonated with triethylamine (150 mg, 1.5 mmol) in chloroform solution at 0-5°C. To the stirred solution of the amine was added cyclohexylisocyanate (175 mg, 1.4 mmol) dropwise and the stirring was continued for 10 hours. The reaction mixture was washed with water, dried ($MgSO_4$) and concentrated under vacuum. On adding petroleum ether dropwise 1-{3-[2-chloroethyl)thio]propyl}-3-cyclohexylurea precipitated as a white solid which was purified by recrystallization from chloroform/petroleum ether 225 mg (62% yield); m.p. 96-98°.

ν_{max} (CH_2Cl_2) 3440, 3360 (NH) 1672.6 (C=O) cm^{-1}.

Pmr ($CDCl_3$) δ 0.9 - 2.1 (m, 12H, CH_2), 2.62 (t, 2H, CH_2), 2.84 (t, 2H, CH_2), 3.23 (q, 2H, CH_2), 3.4 - 3.7 (m, 1H, CH), 3.64 (t, 2H, CH_2), 5.14 (d, 1H, exchangeable, NH), 5.44 (t, 1H, exchangeable, NH). m/e 242 (M-36), Anal. calcd. for $C_{12}H_{23}ClN_2OS$, C = 51.70, H = 8.25, N = 10.05, S = 11.49

Found C = 52.00, H = 8.44, N = 10.04, S = 11.65

A 100 mg (0.36 mmol) portion of the urea was dissolved in 2 ml of 97% formic acid at 0-5°C, and 200 mg (2.9 mmol) of sodium nitrite were added slowly over a 20 min period maintaining a temperature of 0-5°C. After stirring the reaction mixture for 30 min 10 ml of cold water was added. The mixture was extracted with chloroform, the extract was

washed with water, dried ($MgSO_4$), and the solvent was removed affording the nitrosourea 95 as a yellow oil 60 mg (54% yield).

ν_{max} (CH_2Cl_2) 3420 (NH), 1725.5 (C=O). Pmr ($CDCl_3$) δ 0.9 - 2.2 (m, 12H, CH_2), 2.5 (t, 2H, CH_2), 2.8 (t, 2H, CH_2), 3.6 (t, 2H, CH_2), 3.9 (t, 2H, CH_2), 3.7 - 4.1 (m, 1H, CH); 6.8 (d, 1H, exchangeable), m/e 271.1354 (calcd. for $C_{12}H_{21}ClN_3O_2S$, 271.1354, M-HCl). Anal. calcd. C = 46.82, H = 7.15, N = 13.65, S = 10.40

Found C = 46.60, H = 7.15, N = 13.97, S = 10.41

1-{2-[(3-Chloropropyl)thio]ethyl}-3-cyclohexyl-1-nitrosourea 96

S-(3-chloropropyl)thioethylamine hydrochloride 60 was deprotonated with triethylamine and allowed to react with cyclohexyl isocyanate following the procedure described above. 1-{2-[(3-Chloropropyl)thio]ethyl-3-cyclohexylurea was obtained as an off-white solid that was purified by recrystallization from chloroform/petroleum ether (66% yield); m.p. 71°.

ν_{max} (CH_2Cl_2) 3420, 3360 (N-H), 1673.9 (C=O) cm^{-1}.

Pmr ($CDCl_3$) 0.9- 2.15 (m, 12H, CH_2), 2.5 - 2.8 (m, 4H, $2CH_2$), 3.35 (t, 2H, CH_2), 3.65 (t, 2H, CH_2), 3.3 - 3.7 (m, 1H, CH), 4.1 - 4.9 (m, 2H, exchangeable). M^+ (278.1227, calcd. for $C_{12}H_{23}ClN_2OS$, 278.1219).

Anal. calcd. C = 51.70, H = 8.25, N = 10.05, S = 11.49

Found C = 51.84, H = 8.36, N = 9.83, S = 11.63

The urea was nitrosated as described for 95. The nitrosourea 96 was obtained as a yellow oil in 49% yield.

ν_{max} 3400 (NH), 1725.9 (C=O) cm^{-1}.

Pmr ($CDCl_3$), 0.9 - 2.2 (m, 12, CH_2); 2.52 (t, 2H, CH_2), 2.7 (t, 2H, CH_2), 3.6 (t, 2H, CH_2), 4.0 (t, 2H, CH_2), 3.7 - 4.1 (m, 1H, CH), 6.84 (d, 1H, exchangeable), M^+ 307.1123 (calcd. for $C_{12}H_{22}ClN_3O_2S$, 307.1121).

Anal. calcd. for N = 13.65, S = 10.40

Found N = 13.51, S = 10.43

METHODS

Fluorescence Assay for Determining CLC Sequences in DNA Produced by Triazenes and Nitrosoureas

A 20 µℓ aliquot was taken at intervals from the reaction mixture (50 mM potassium phosphate, pH 7.2; 1.0 A_{260} units of λ-DNA; 5 mM drug; total volume, 200 µℓ) at 37°C and added to the standard assay mixture (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 µg/ml of ethidium). The fluorescence after the heat denaturation and cooling cycle compared with control times 100 gives the percentage of CLC-DNA in a sample.

Detection of Type I and Type II SSS of DNA

The SSS of DNA was studied by fluorometric methods using ethidium bromide assay as described in Chapter III. Relaxed PM2-CCC-DNA 1.0 A_{260} was incubated with a 5 mM concentration of the desired drug.

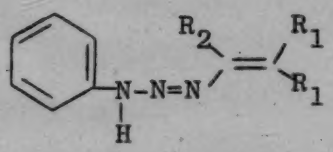
General Method for the Aqueous Decomposition of Nitrosoureas

All decompositions and analyses of the volatile decomposition products were performed following the procedure given in Chapter IV.

CHAPTER VI.

ARYLVINYLTRIAZENES AND THEIR REACTIONS WITH DNA

Vinyltriazenes 98a-d have been suggested as sources of vinyl cations.[112] It was considered desirable to investigate the chemistry and the biochemical aspects of this class of compounds. A previous study on vinyltriazenes[112]



98-a, $R_1=R_2=C_6H_5$
98-b, R_1=p-tolyl; $R_2=C_6H_5$
98-c, $R_1=C_6H_5$; $R_2=CH_3$
98-d, $R_1=C_6H_5$; $R_2=H$

dealt with the triazenes extensively substituted in the vinyl group. The present study has been mainly directed to the triazenes unsubstituted in the vinyl group for the following reasons:

1 A vinyl cation has been suggested as a possible intermediate in the aqueous decomposition of antitumor 2-haloethylnitrosoureas (Figure 15). Therefore, it was desirable to generate vinyl cations from an independent source such as a vinyltriazene and study their chemical reactivity towards biological macromolecules, principally DNA.

2 For a given aryl group of an arylvinyltriazene any substitution in the vinyl moiety reduces the solubility of a triazene in aqueous solutions. Since the *in vitro*

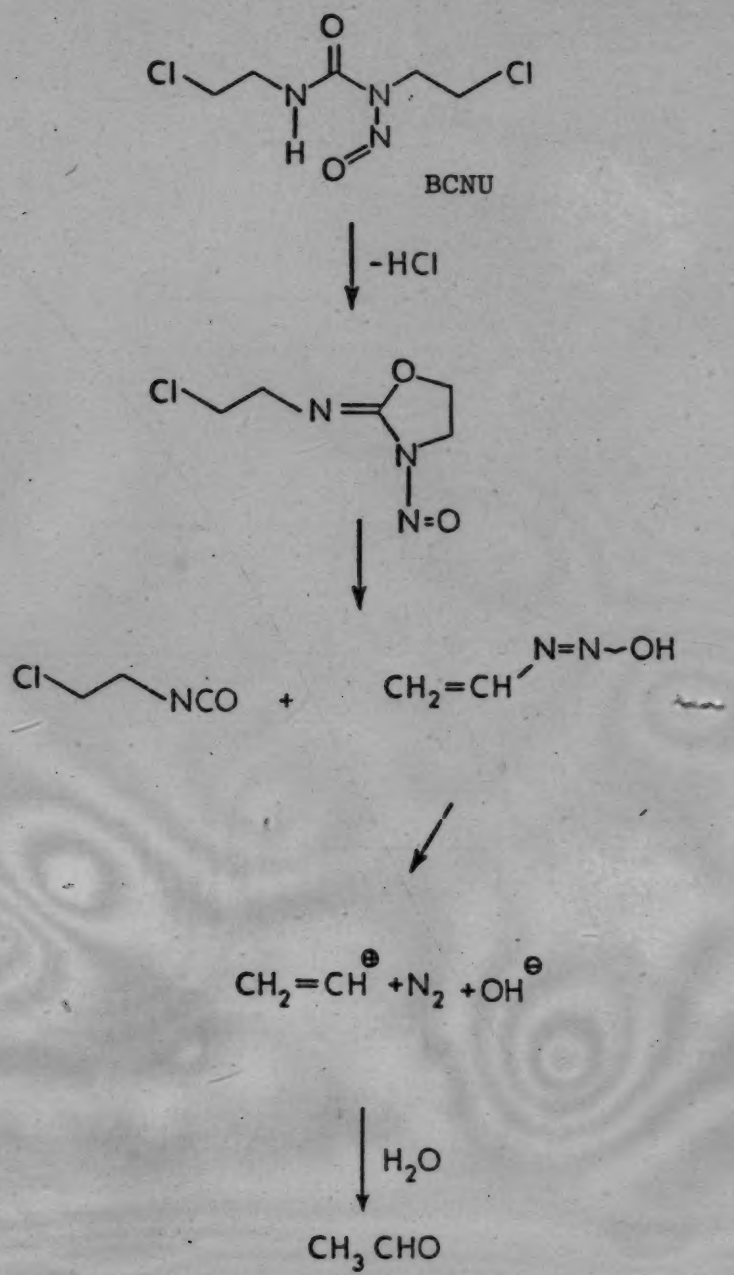


Figure 15. "Abnormal" decomposition suggested for bis(2-chloroethy)nitrosourea (BCNU).

reactions with DNA are conducted under physiological conditions, the aqueous solubility of a compound under investigation becomes an important aspect of its biochemical properties.

Synthesis and Properties of Arylvinyltriazenes

The arylvinyltriazenes employed in this study were prepared by coupling arylazides with vinylmagnesiumhalides [Equation 5]. In certain cases this procedure has been modified by substituting a vinyllithium for the Grignard reagent [Equation 6].

$$ArN_3 + RCH{=}CHMgX \longrightarrow [ArN(MgX){-}N{=}N{-}CH{=}CHR] \xrightarrow{H_2O} Ar{-}NH{-}N{=}N{-}CH{=}CHR \quad [5]$$

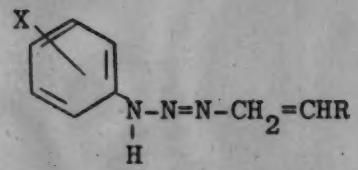
$$ArN_3 + RCH{=}CHLi \longrightarrow [ArN(Li){-}N{=}N{-}CH{=}CHR] \xrightarrow{H_2O} Ar{-}NH{-}N{=}N{-}CH{=}CHR \quad [6]$$

X = Cl, Br

Although the vinylmagnesiumhalides condensed with p-cyanophenylazide without significant interference by attack on the nitrile group as observed earlier (see Chapter III), the corresponding vinyllithiums seemed to attack the nitrile group which limited their application. Several vinyltriazenes have been synthesized by employing these two routes (Table 15).

The only example of an arylvinyltriazene unsubstituted in the vinyl group 99 was isolated during an attempted synthesis of the triazoline 100 [Equation 7].[113] This reaction is severely limited in scope since other azides give rise

TABLE 15

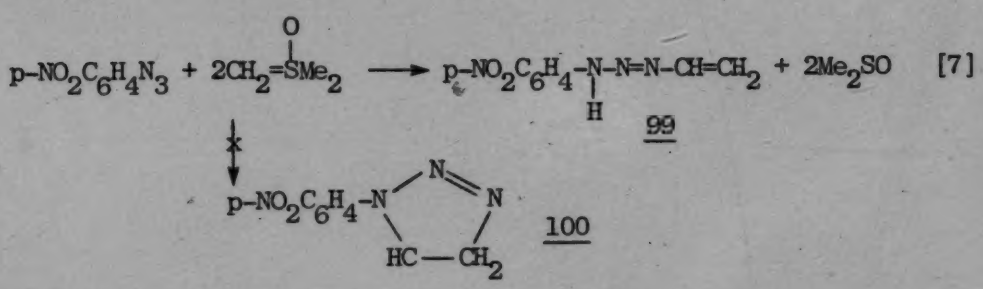


X	R	Compound	
H	H	1-phenyl-3-vinyltriazene	101
p-CN	H	1-(p-cyanophenyl)-3-vinyltriazene	102
p-Cl	H	1-(p-chlorophenyl)-3-vinyltriazene	103
p-OMe	H	1-(p-methoxyphenyl)-3-vinyltriazene	104
O-Me	H	1-(o-methoxyphenyl)-3-vinyltriazene	105
2,5-di-OMe	H	1-(2,5-dimethoxyphenyl)-3-vinyltriazene	106
H	Me	1-phenyl-3-(1'-propenyl)triazene	107

Table 15, continued

X	R	Compound	
p-OMe	Me	1-(p-methoxyphenyl)-3-(1'-propenyl)triazene	108
O-OMe	Me	1-(o-methoxyphenyl)-3-(1'-propenyl)triazene	109
		1-phenyl-3-ethylidenetriazene	110
		1-(2,5-dimethoxyphenyl)-3-ethylidenetriazene	111

to the corresponding triazolines.



[7]

99

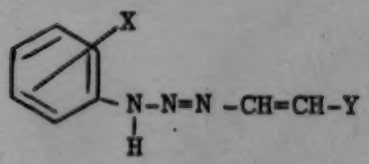
100

It has been commonly experienced that in order to obtain a stable monoalkyltriazene an electron-withdrawing group in the phenyl ring, particularly at the p-position is essential. However, an extended conjugation provided by the vinyl group imparts an unusually high stability to the system and it has been possible to isolate vinyltriazenes with substituents on the phenyl ring varying from p-CN (strongly electron-withdrawing) to p-oMe (strongly electron-releasing) groups. The stability of vinyltriazenes seems to correlate with the substituent-effect observed in general for monoalkyltriazenes. Vinyltriazenes with an electron-withdrawing group on the phenyl ring are more stable than if the substituent is electron-releasing as indicated by the respective rates of decomposition measured polarographically in Table 16.

Vinyltriazenes like other monoalkyltriazenes can not be purified by standard chromatographic methods owing to their rapid degradation on solid adsorbents. The solid vinyltriazenes were purified by recrystallization. The compositions,

TABLE 16

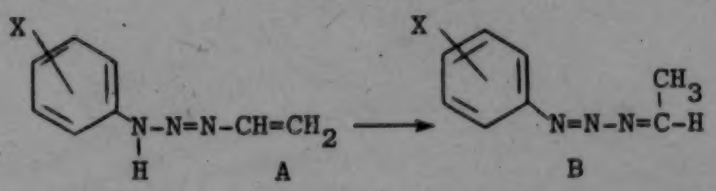
HALF-LIFE ($t_{\frac{1}{2}}$) OF ARYLVINYLTRIAZENES DETERMINED POLAROGRAPHICALLY IN PHOSPHATE BUFFERED (0.01 M, pH 7.1) (95:5 AQUEOUS ACETONITRILE AT 37.5°



No. Triazene	X	Y	Reduction Potential	$t_{\frac{1}{2}}$ (sec).
101	H	H	-1.045	1536
102	p-CN	H	-0.922	7710
103	p-Cl	H	-0.956	1734
104	p-OMe	H	-0.993	588
106	2,5-di-OMe	H	-0.015	3612
108	p-OMe	CH_3	-1.020	324
109	o-OMe	CH_3	-0.980	234

structures and purity of the present series of triazenes were established by spectroscopic measurements. The pmr spectra showed that the vinyltriazenes were obtained in an acceptably pure state and did not require further purification.

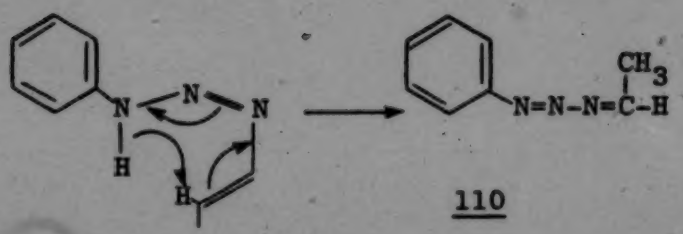
Unlike other monoalkyltriazenes, the vinyltriazenes exist in solution only in tautomeric form A. Conversion of A to B



X = p-CN, p-Cl, H,
p-OMe; o-OMe; 2,5 di-OMe

appears to be irreversible. When triazene 101 was subjected to distillation under vacuum, in addition to its thermal decomposition, the isomerization from 101 to 110 took place. A similar isomerization was observed when 2,5-dimethoxyphenylazide was condensed with vinylmagnesiumchloride in the presence of magnesium bromide generated *in situ* under anhydrous conditions. The isomeric product 111 was obtained in a quantitative yield. A concerted [1,5] sigmatropic migration of hydrogen may be involved in the isomerization of 101 to 110 (Scheme 25).

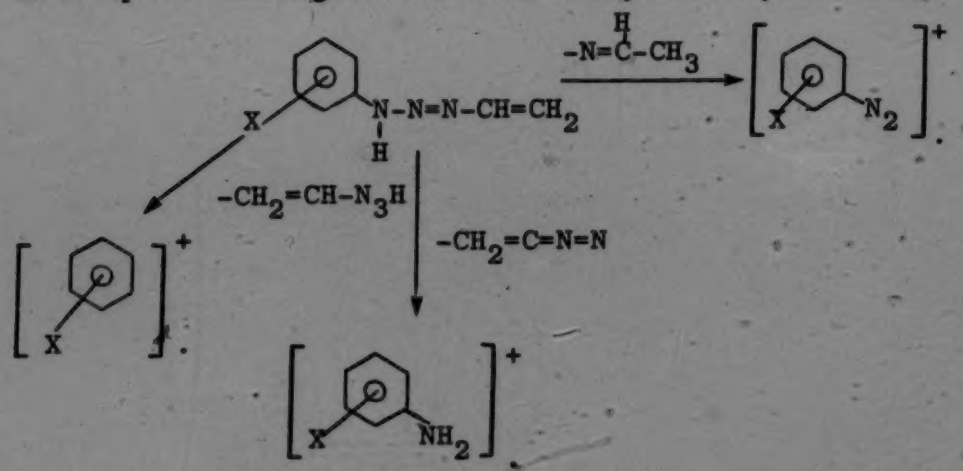
Scheme 25



110

The fragmentation patterns in the mass spectra of vinyltriazenes are similar to those of other monoalkyltriazenes reported in previous chapters. Vinyltriazenes like other monoalkyltriazenes are acid labile which is reflected in their rates of aqueous decomposition measured polarographically [Table 17].

Mass Spectral Fragmentation of 1-Aryl-3-Vinyltriazenes

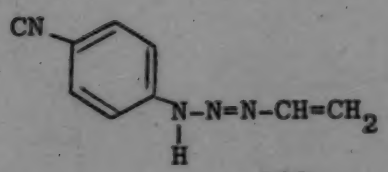


Decomposition of the Vinyltriazenes

Studies on the aqueous decomposition of monoalkyltriazenes reported in previous chapters were essential in order to interpret their mode of action on DNA. A similar

TABLE 17

VARIATION OF HALF-LIFE ($t_{\frac{1}{2}}$) OF A VINYLARYLTRIAZENE DETERMINED POLAROGRAPHICALLY AS A FUNCTION OF SOLUTION pH AT 37.5°



102

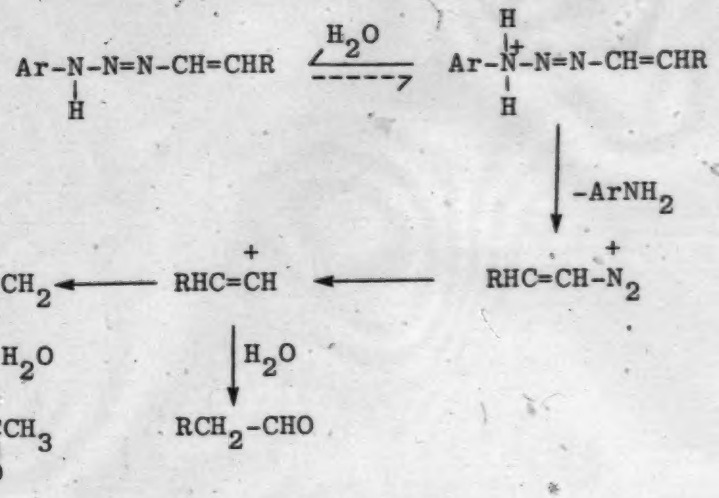
pH	$t_{\frac{1}{2}}$ (sec).
5.0	1152
5.8	5214
7.1	7710

study was conducted on selected vinyltriazenes 101, 109 and 110. The compounds were allowed to decompose at 37° in phosphate buffered (0.1 M, pH 7.2) aqueous solution in gas-tight vials and the volatile products were analyzed by GC and identified by GC/MS. The involatile products were separated by chromatography and identified spectrophotometrically. Thus, 1-phenyl-3-vinyltriazene 101 and its isomer 110 both gave acetaldehyde accounting for 100% of the volatiles. Aniline was the other common product from 101 and 110. The triazene 109 afforded propionaldehyde and acetone in the ratio 10:1 respectively. o-Anisidine accounted for 95% of the triazene 109.

Identification of acetaldehyde and aniline from 101 as products of aqueous decomposition does not necessarily indicate the intermediacy of vinyl cation since its isomer 110 also yields the same products. However, detection of acetone in addition to propionaldehyde as a product of aqueous decomposition of 109 supports the intermediacy of a vinyl cation (Scheme 26). An S_N2-type nucleophilic displacement at an sp^2 hybridized carbon is unlikely, therefore, the formation of vinyl cations from vinyltriazenes in general is plausible.

The vinyltriazenes studied in this chapter give a positive test of alkylation with NBP (see chapter IV for introduction to the reagent NBP).

SCHEME 26



R = H, CH_3

Action of Vinyltriazenes on DNA

Arylvinyltriazenes alkylate PM2-CCC-DNA under physiological conditions as demonstrated by the ethidium fluorescence assay. The rate of alkylation parallels the observed rate of decomposition of vinyltriazenes measured polarographically in Table 18.

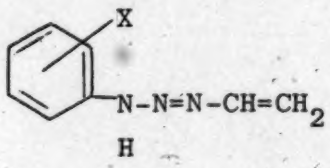
DNA Single Strand Breaks Induced by Arylvinyltriazenes and the Nature of its Origin

A production of SSS was observed when a triazene treated PM2-CCC-DNA was incubated at pH 7, 37° and assayed at pH 11.8 buffer.

Unlike other monoalkyltriazenes discussed in the previous chapters, vinyltriazenes show both types of single strand scission. The origin of Type I SSS observed with 2-hydroxyethyltriazenes has been related to hydroxyethylation of the phosphate residues of DNA (see Chapter III). With arylvinyltriazenes the Type I SSS is dependent on the nature of the substituent on the phenyl ring. When the triazenes 101, 102 and 104 were allowed to react with DNA under physiological conditions the rate of strand scission was parallel to the observed rate of strand scission of AP DNA by the corresponding aromatic amines under similar conditions (Figure 16). On the basis of the latter observation it may be concluded that the Type I SSS observed with the vinyltriazenes is mainly due to base alkylation leading to

TABLE 18

RELATIVE EXTENTS OF ALKYLATION OF DNA AND RATES OF AQUEOUS DECOMPOSITION OF ARYLVINYLTRIAZENES



Compound	X	% Loss of Fluorescence 37°, pH 7.1 in 30 min	$t_{\frac{1}{2}}$ (sec)[2]
102	p-CN	0	7710
101	H	33	1536
104	p-OMe	53	588

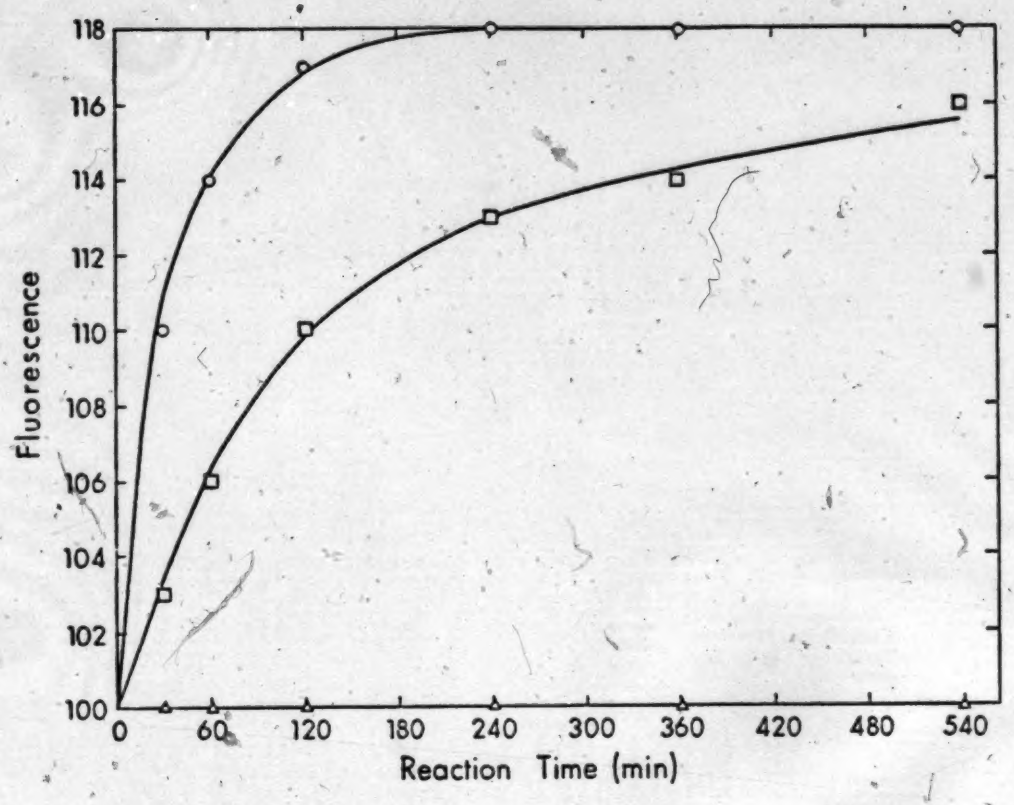


Figure 16. Reaction of AP PM2-CCC-DNA incubated at 37°. pH 7.2 with: 5 mM (O) p-methoxyaniline; (□) aniline; (Δ) p-aminobenzonitrile, or control. Fluorescence values obtained within 30 sec of addition of 20 µℓ aliquot to pH 11.8 assay solution.

depurination and depyrimidination followed by Schiff base formation[79,80] with the released aromatic amines. This further results into strand degradation (Figure 16). The Type I SSS observed with 104 was rapid and extensive which prevented the detection of the Type II SSS. However, the Type II SSS was confirmed in the case of the less reactive triazene 106 employing basic as well as enzymatic hydrolyses (see Chapter III). The vinyltriazenes were inactive *in vivo* in standard animal leukemia test systems.

EXPERIMENTAL

1-Phenyl-3-vinyltriazenes

General Procedure

A solution of the aromatic azide (10 mmol) in anhydrous ether (2-4 ml) was added slowly to a stirred solution of vinylmagnesium bromide in tetrahydrofuran (THF) (11 mmol) over a period of 30 min. After stirring for another 30 min. 5 ml of cold water was added followed by a saturated aqueous solution of ammonium chloride just enough to dissolve all the solid. The aqueous solution was extracted in ether (2 x 10 ml). The ether layer was washed with water and dried ($MgSO_4$). On evaporating the solvent the vinyltriazenes were left behind in acceptably pure state. Any attempt to purify liquid triazenes by preparative chromatography on silica, florisil or alumina was unsuccessful owing to rapid decomposition on these surfaces. The solid products were recrystallized from CH_2Cl_2 at a low temperature.

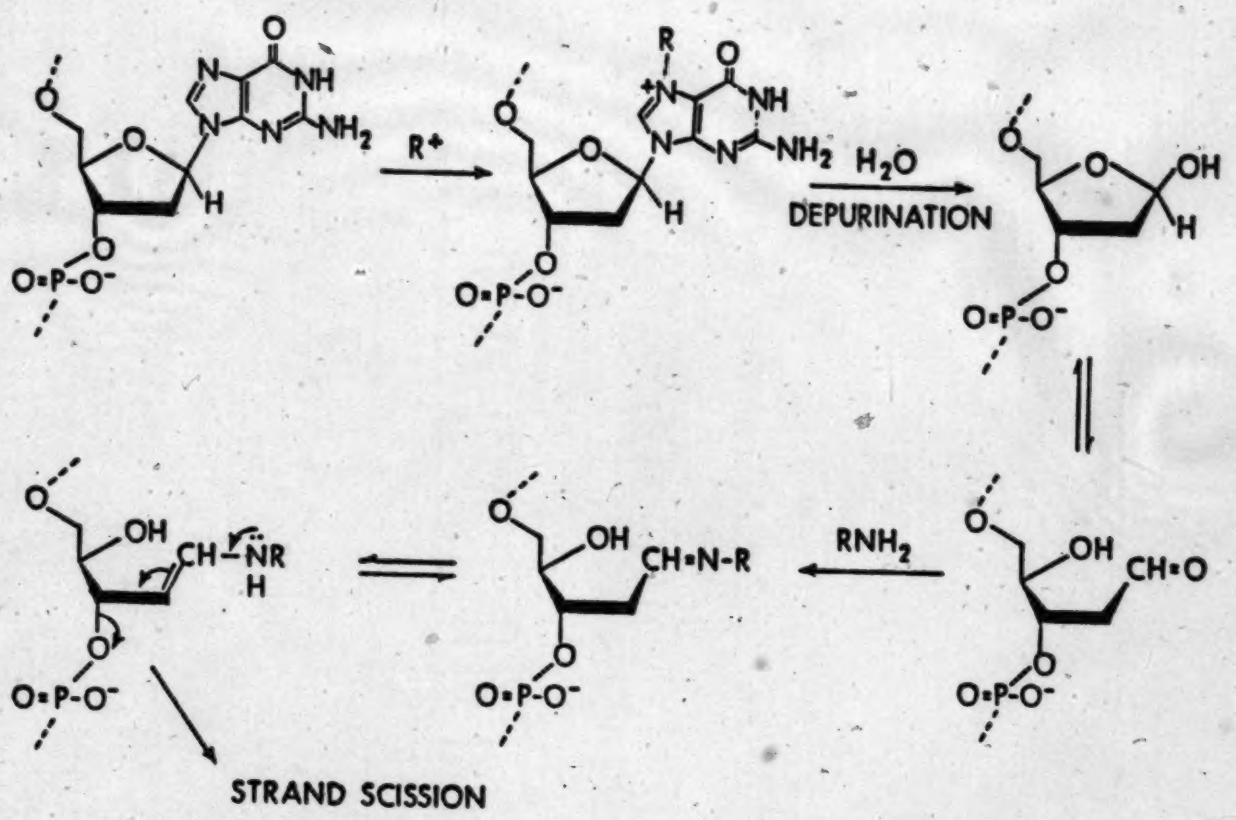


Figure 17. Suggested mechanism for amine catalyzed transformation of an apurinic site resulting from DNA alkylation, to a single strand internucleotide break.

This procedure afforded the following triazenes.

1-Phenyl-3-Vinyltriazene 101

Viscous liquid; pmr ($CDCl_3$ - 45°) δ4.85 (d, 1H); 5.15 (d, 1H); 6.7 (m, 1H); 7 - 7.4 (m, 5H); 11.14 (br, 1H, exchangeable); M^+, 147.0793 (38.42%), (calcd. for $C_8H_9N_3$; 147.0797); m/e 119.0715 (2.33%) ($M-N_2$, calcd. for $C_8H_9N_2$, 119.0736); 93.0577 (100%) aniline. ν_{max} 3300, 1733 and 1600 cm^{-1}.

1-(p-Cyanophenyl)-3-vinyltriazene 102

Yellow solid, m.p. 120°; pmr ($CDCl_3$) δ5.15 (d, 1H), 5.45 (d, 1H), 7.1 - 7.4 (m, 3H), 7.6 (m, 2H), 9.55 (br, 1h, exchangeable); M^+ 172.0750 (28.37%) (calcd. for $C_9H_8N_4$, 172.0750); m/e 144.0685 (3.87%) ($M-N_2$, calcd. for $C_9H_8N_2$, 144.0687); 102.0343 (100%); ν_{max} 3253, 2200, 1600 cm^{-1} and 1590 cm^{-1}.

1-(p-Chlorophenyl)-3-Vinyltriazene 103

Yellow solid m.p. 68°; pmr ($CDCl_3$) δ 4.75 (d, 1H), 5.0 (d, 1H), 7.0 - 7.3 (m, 5H), 9.6 (br, 1H, exchangeable); M^+ 181.0104 (45.90%); m/e 153.0339 (0.95%), 127.0184 (p-chloroaniline, calcd. for C_6H_6NCl, 127.0170); ν_{max} 3160, 1680.

1-(p-Methoxyphenyl)-3-Vinyltriazene 104

(A low melting hygroscopic solid); pmr ($CDCl_3$ -30°) δ 3.75 (s, 3H), 4.5 (d, 1H), 4.75 (d, 1H), 6.8 - 7.45 (m, 5H), 9.0 (br, 1H, exchangeable); M^+ 177.0899 (39.44%), (calcd. for $C_9H_{11}N_3O$, 177.0902), 149.0830 (1.71%) ($M-N_2$, calcd. for ($C_9H_{11}NO$ 149.0841), 107.0505 (100%); ν_{max} 3280, 1720 and 1600 cm^{-1}.

1-(o-Methoxyphenyl)-3-Vinyltriazene 105

(Viscous liquid); pmr ($CDCl_3$ - 30°) δ 3.75 (s, 3H), 5.05 (d, 1H), 5.4 (d, 1H), 6.7 - 7.55 (m, 5H), 9.75 (br, 1H, exchangeable); M^+ 177.0902 (32.54%) (calcd. for $C_9H_{11}N_3O$, 177.0902), m/e 149.0852 (0.55%) ($M-N_2$, calcd. for $C_9H_{11}NO$ 149.0863), 123.0684 (100%) o-anisidine; ν_{max} 3330 and 1600 cm^{-1}.

1-(2,5-Dimethoxyphenyl)-3-Vinyltriazene 106

(Viscous liquid); pmr ($CDCl_3$ -30°), δ 3.75 (d, 6H), 5.1 (d, 1H), 5.45 (d, 1H), 6.45 (γ, 1H), 6.75 (d, 1H), 7.1 - 7.35 (m, 2H), 9.7 (br, 1H, exchangeable), M^+ 207.1005 (43.26%) (calcd. for $C_{10}H_{13}N_2O_2$, 207.1008), m/e 179.0945 (0.74%), ($M-N_2$, calcd. for $C_{10}H_{13}NO_2$, 179.0946), 153.0790 (100%), 2.5-dimethoxyaniline; ν_{max} 3320 and 1600 cm^{-1}.

The following isomeric mixtures of propenyltriazenes were prepared by the following general procedure.

20 Mmol of 1-bromo-1-propene(cis-trans mixture) was taken in 15 ml of dry ether. To the solution was added 200 mmol of freshly cut lithium after thoroughly washing with petroleum ether. The reaction mixture was stirred at room temperature under anhydrous conditions. A vigorous reaction started in about 5 minutes. After stirring for 20 min the reaction mixture turned slightly yellowish indicating complete lithiation of the bromoalkene. To the vinyllithium was added 4 mmol of aryl azide diluted with 4 ml of dry ether at room temperature. After stirring the reaction mixture for 20 min any unreacted lithium was removed mechanically and the cooled reaction mixture was treated with dilute ammonium chloride solution. The ether layer was removed, washed with cold water and dried ($MgSO_4$). On evaporating ether the vinyltriazenes separated in a reasonably pure state. The following three triazenes prepared by this procedure were formed as viscous liquids and could not be further purified.

1-(Phenyl)-3-(1'-propenyl)Triazene 107

(Viscous liquid). pmr ($CDCl_3$ -35°) δ 1.9 (d, 3H), 5.2 - 6.25 (2m, 1H), 6.9 - 7.5 (m, 6H), 10.25 (s, 1H, exchangeable); M^+ 161.0949 (39.56%) (calcd. for $C_9H_{11}N_3$ 161.0953), m/e 133.0871 (2.43%) ($M-N_2$, calcd. for $C_9H_{11}N$,

133.0892), 93.0577 (100%), (aniline, calcd. for C_6H_7N, 93.0578).

1-(p-Methoxyphenyl)-3-(1-propenyl)Triazene 108

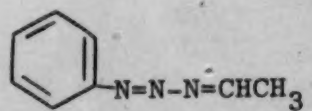
(Viscous liquid). pmr ($CDCl_3$) δ 1.7 - 1.85 (2d, 3H), 3.7 (s, 3H), 4.8 - 5.9 (2m, 1H), 6.7 - 7.4 (m, 5H), 9.4 (br, 1H, exchangeable); M^+ 191.1055 (calcd. for $C_{10}H_{13}N_3O$ 191.1059), 163.0986 (1.71%) ($M-N_2$, calcd. for $C_{10}H_{13}NO$ 163.0997), 123.0684 p-anisidine, 107.0505 (100%).

1-(o-Methoxyphenyl)-3-(1'-propenyl)Triazene 109

(Viscous liquid). (pmr, $CDCl_3$, -35°), 1.8 - 2.1 (2d, 3H), 3.8 (s, 3H), 5.3 - 6.3 (2m, 1H), 6.8 - 7.2 (m, 4H, aromatic), 7.55 (m, 1H), 9.7 (s, 1H, exchangeable), M^+ 191.1056 (25.58%) (calcd. for $C_{10}H_{13}N_3O$, 191.1059), 163.0988 (1.38%), ($M-N_2$, calcd. for $C_{10}H_{13}NO$ 163.0997), 123.0629 (100%) o-anisidine. ν_{max} 3333 and 1600 cm^{-1}.

Isomerization of 101 → 110

Triazene 101 was distilled under vacuum (.2 mm) and the fraction at ~100°C was collected. The product was identified as 1-phenyl-3-ethylidenetriazene 110. The compound was identified by its distinctive pmr spectrum.



110

pmr ($CDCl_3$) δ 2.15 (d, 3H), 6.5 - 7.4 (m, 5H), 7.8 (Q, 1H), m/e, 119.0734 ($M-N_2$, calcd. for C_8H_9N 119.0735), 93.0435 (100%) aniline.

SUMMARY

This study has examined a number of aspects of the chemistry of several types of monoalkyltriazenes. Following from our conclusions in the first phase of the study new types of alkyltriazenes were synthesized incorporating structural features which have resulted in significantly increased antineoplastic activity in animal test systems. Polarography has proved to be a convenient technique for measuring the relative stabilities and rates of decomposition of the triazenes in aqueous solutions. The increase in rates of decomposition of triazenes on lowering the pH of the medium suggests that the monoalkyltriazenes may exhibit some selectivity for tumor tissues that exhibit slightly lower pH than the normal tissue. 2-haloethyl alkylating species have been detected in the decomposition of 2-haloalkyltriazenes. Evidence was also obtained for the presence of a triazoline intermediate from a 2-chloroethyltriazene during aqueous decomposition. Some evidence for the presence of an episulfonium species has been obtained in the aqueous decomposition of S-(2-chloroethyl)thioethyltriazenes. Indication of a parallel pathway possibly involving a triazoline type intermediate was also obtained.

The essential structural features necessary to promote DNA interstrand cross-linking which empirically appears to enhance their antileukemic properties have been delineated by model studies.

The reactions of triazenes with purified DNA have also been studied. In addition to alkylation, S-(2-chloroethyl)-thioalkyltriazenes that generate bifunctional alkylating agents produce DNA interstrand cross-links as evidenced by ethidium fluorescence assay. The studies indicate a preference for alkylation of phosphate residues of DNA by monoalkyltriazenes.

DNA degradation by monoalkyltriazenes of the following structural types has also been examined where triazene induced single strand scission (SSS) depends on the nature of the alkyl group. Type I SSS which results from phosphate alkylation is extensive in the case of hydroxyethyltriazenes while 2-haloethyltriazenes appear to react largely via Type II SSS of DNA by base alkylation followed by depurination or depyrimidination and subsequent hydrolysis of the AP sites.

Using vinyltriazenes it was possible to investigate the chemical properties under physiological conditions and the possible biological significance of vinyl cations which have been previously postulated as intermediates in the aqueous decomposition of 2-haloethylnitrosoureas, and of the aromatic amines which are generated from aqueous decomposition of aryltriazenes. Only the aromatic amines with

electron-releasing groups appear to exhibit activity towards formyl groups at DNA AP sites via Schiff bases which may be of biological significance.

The results highlight significant differences between the underlying chamistry of the monoalkyltriazenes and the structurally related nitrosoureas. These differences may reflect the different modes of action *in vivo* of the two classes of compound.

APPENDIX

DNA STRUCTURAL FEATURES

Since the basis of the various assays employed in this study is the ability of ethidium bromide to intercalate in the duplex regions of double helical DNA, it is important to discuss briefly the nature of DNA for the proper understanding of the mechanism of action of the drugs.

In 1953, Watson and Crick described DNA (B form) as a double helix having two antiparallel polynucleotide chains.[119] The most significant features of the proposed structure of DNA are shown in Figure 18. DNA is partially composed of four aromatic heterocyclic bases: adenine (A), guanine (G), thymine (T) and cytosine (C). These four bases are linked together by β-glycosidic linkage to the C-1' carbon of the deoxyribose sugar to form the four corresponding nucleoside moieties. The two free hydroxy groups on the deoxyribose sugar are esterified with phosphoric acid to yield nucleotides. These nucleotides are connected together to form a sequence of nucleotides joined through phosphodiester bonds at both the C-3' and C-5' hydroxy groups of the deoxyribose. The hydrogen bonded base pairs form a plane which is 3.4 Å in thickness. Each base pair is aligned approximately 36° relative to the base pair immediately below it. The double helix makes a complete turn every 34 Å, and each turn contains approximately ten base pairs. The phosphate groups on the back-

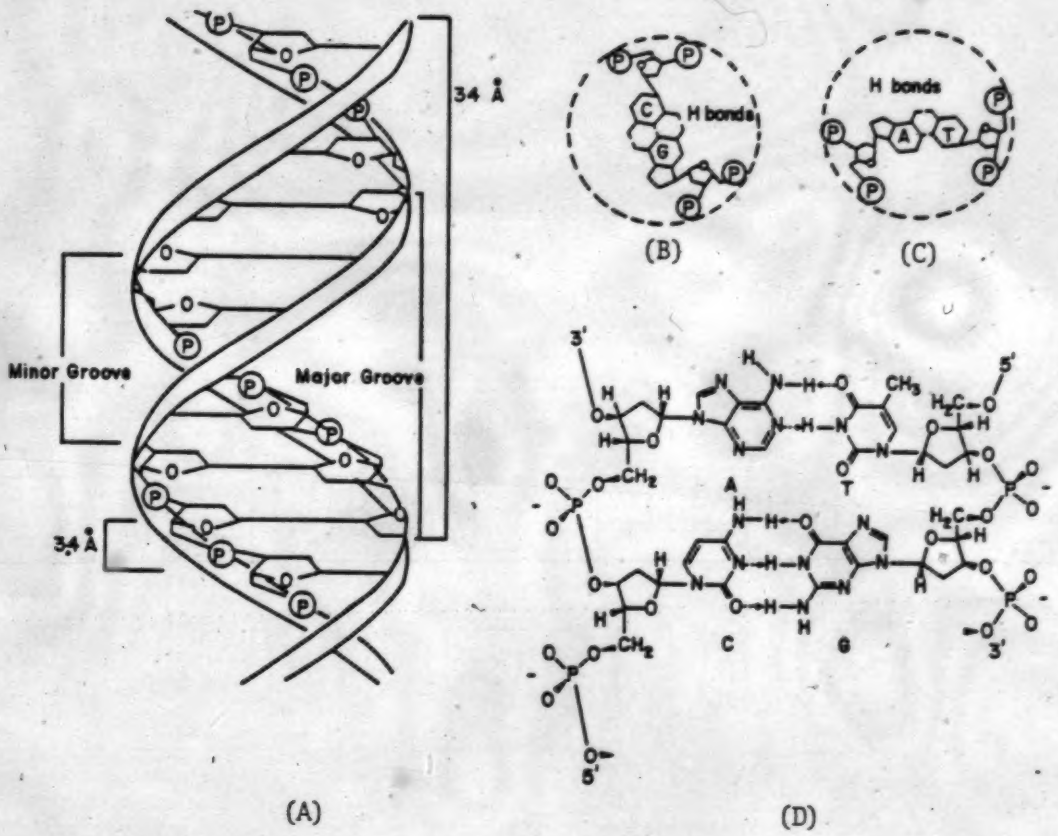


Figure 18. Schematic Representations of the Hydrated "B" Form of the DNA Molecule. (A) depicts a side view of a segment of the double helix which is composed of two helically wound ribbons connected by dark lines (base pairs) to the deoxyribose phosphate backbone. (B) and (C) represent GC and AT base pairs respectively. (D) represents a distorted (flattened) view of a DNA segment.

bone make DNA highly negatively charged and allow it to interact with molecules containing positive charges. The sugar-phosphate chains as shown in Figure 18 are separated by about 120° which creates the alternating major and minor grooves along the axis of the double helix.

The PM2-CCC-DNA consists basically of a closed circular covalently-linked DNA double helix coiled in a right-handed fashion (negatively supercoiled). When an intercalator interacts with the base pairs of DNA, the neighboring base pairs are locally unwound and separated to create a space for the insertion of the intercalator (Figure 19). In the case of PM2-CCC-DNA the process of intercalation results in unwinding of the native right-handed superhelical turns until the strands no longer have a superhelical structure. The free energy decrease from the superhelix uncoiling is added to the free energy of drug binding. If more molecules are intercalated, the superhelix is forced to rewind to form a left-handed superhelical structure (positively supercoiled) and part of the free energy released is expended to compensate for the free energy associated with the reversed supercoil formation (Figure 20).

The amount of rotation of base pairs from the native position is defined as the intercalation unwinding angle. The unwinding angle determined for ethidium bromide is 26°.[120]

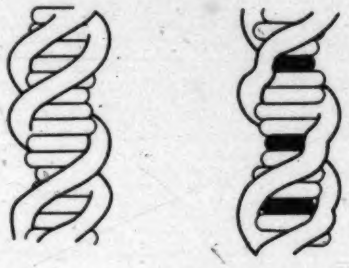


Figure 19. Native DNA (left) and DNA containing intercalated molecules (right).

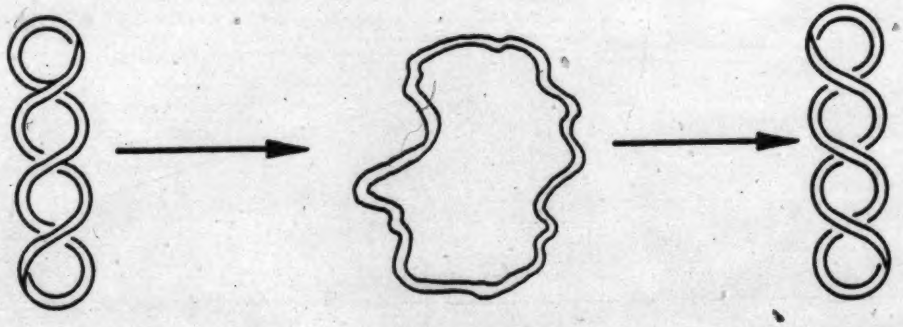


Figure 20. Schematic Representation of the Unwinding Process of CCC-DNA.

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